



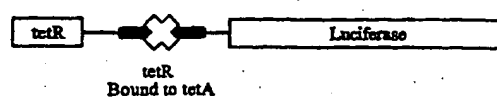
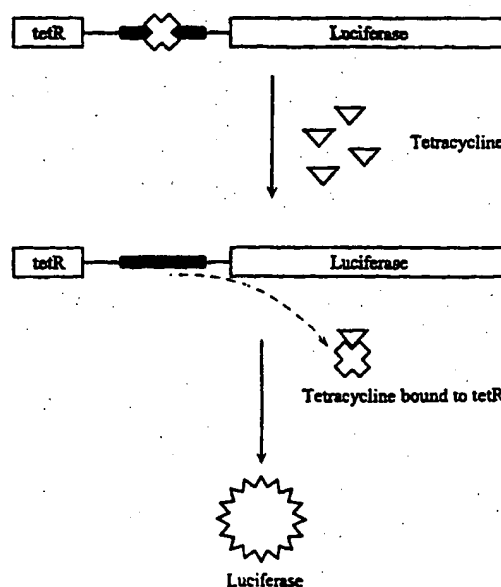
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(54) Title: TETRACYCLINE ASSAY METHOD

(57) Abstract

The invention relates to a method for the determination of a tetracycline in a sample. The method is characterized in that the sample is brought into contact with prokaryotic cells encompassing a DNA vector including a nucleotide sequence encoding a light producing enzyme under transcriptional control of a tetracycline repressor and a tetracycline promoter, detecting the luminescence emitted from the cells, and comparing the emitted luminescence to the luminescence emitted from cells in a control containing no tetracycline. The invention also concerns recombinant prokaryotic cells capable of emitting light in response to the existence of a tetracycline in a sample. Furthermore, the invention relates to novel DNA vectors useful for the construction of said prokaryotic cells.

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Tetracycline assay method.

FIELD OF THE INVENTION

This invention relates to a method for the determination of a tetracycline in a
5 sample. The invention also concerns recombinant prokaryotic cells capable of
emitting light in response to the existence of a tetracycline in a sample. Furthermore,
the invention relates to novel DNA vectors useful for the construction of said
prokaryotic cells.

10 BACKGROUND OF THE INVENTION

The publications and other materials used herein to illuminate the background of the
invention, and in particular, cases to provide additional details respecting the
practice, are incorporated by reference.

15 Whole cells can be used in methods based on the use of living cells or organisms as
sensor tools of detection. Many of these methods utilize bacterial or yeast cells.
Prokaryotic organisms and especially *Escherichia coli* bacterium are very well
characterized and maps of genes and their sequences at nucleotide level are known.
Therefore the behavior of the whole cell sensor can be better understood. Because
20 of this fact it is also possible to develop analyte or group specific sensors utilizing
different regulatory regions of genomes and also various microbial strains.

Whole cells can be utilized in biosensors which are devices consisting of 1) a
sensor, 2) a recording unit and 3) a possible connector such as fiber optic guide
25 between 1 and 2. The recording unit has several choices of what is the physical
background of the measurement. It can be change in heat, conductance, color
reaction, changes in fluorescent properties, emission of endogenous light from the
sensor cells etc.

Antibiotics used as medicines against microbial invasion are detected from body fluids in order to study the dosage and penetration of the medicine. Often the effective therapeutic range of the antibiotic is rather narrow and the risks of
5 overdosage might be too big. It is also important to measure the presence or concentration of antibiotics from meat and milk due to syndrome of allergic people. In the course of cheese production milk used as starting material should not contain antibiotics due to the fact that cheesemaking bacteria are not able to work on contaminated milk.

10

Conventional tests for the measurement of toxic substances such as antimicrobial agents (antibiotics) are based on the inhibition of growth. Growth inhibition can be followed by monitoring the zone where the growth of microbes is inhibited on a nutrient agar plate around a disk onto which an antibiotic dilution was pipetted.
15 Typical examples of agar diffusion tests are cylindrical, hole or disk methods. The difference in these tests is only restricted in the way the sample is applied on the agar and also the way the bacteria in the test is used. Another means is to follow the metabolism of the test organisms by estimating the intensity of a color reaction which is affected by the inhibitory antibiotic present and comparing it to the
20 uninhibited control (e.g. the commercial products: Delvo TestTM, Brilliant black-reduction test, Charm Farm Test, Charm AIM-96 and Valio T101-test). Since microbiological methods utilize bacteria or their spores it is the sensitivity of the test bacteria which is of utmost importance. Thus far one had to make compromises in the choice of a suitable test strain since great sensitivity against antimicrobial agents
25 and other characteristics needed for the test strain have not been common features for the same strain of bacteria. A major drawback when using microbes in antibiotic residue tests is slow and unsensitive performance. Since in these methods one always controls in a way or other the growth of the tester strain one cannot imagine

the test to be performed in an hour. This is due to the fact that the growth of the microbe is a slow phenomenon even at its fastest mode. Also in many cases microbes are in spores or freeze-dried, the regeneration of which makes the tests even more slow to perform.

5

OBJECT AND SUMMARY OF THE INVENTION

The object of the invention is to provide a novel method of determining a tetracycline in a sample where said method is rapid and selective for tetracyclines, i.e. the method is able to distinguish tetracyclines from other antimicrobial agents.

10

According to one aspect of the invention a method for the determination of a tetracycline in a sample is provided, wherein the method is characterized in that

- the sample is brought into contact with prokaryotic cells encompassing a DNA vector including a nucleotide sequence encoding a light producing enzyme under
- 15 transcriptional control of a tetracycline repressor and a tetracycline promoter,
- detecting the luminescence emitted from the cells, and
- comparing the emitted luminescence to the luminescence emitted from cells in a control containing no tetracycline
- wherein a detectable luminescence higher than a luminescence of the control
- 20 indicates the presence of tetracycline in the sample.

According to another aspect, the invention concerns a recombinant prokaryotic cell which encompasses a DNA vector including a nucleotide sequence encoding a light producing enzyme, tetracycline repressor and tetracycline promoter.

25

According to yet another aspect, the invention concerns a plasmid which comprises either

- the luxCDABE genes (SEQ ID NO: 3), tetracycline repressor (TetR) (SEQ ID NO: 11) and tetracycline promotor (TetA) (SEQ ID NO: 9) from *Tn10*, or
- the insect luciferase gene (SEQ ID NO: 1), tetracycline repressor (TetR) (SEQ ID NO: 11) and tetracycline promotor (TetA) (SEQ ID NO: 9) from *Tn10*.

5

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1a shows schematically the method according to this invention, where cells cloned with the plasmid pTetLux1 (SEQ ID NO: 3) are used.

- 10 Figure 1b shows schematically the method according to this invention, where cells cloned with the plasmid pTetLuc1 (SEQ ID NO: 1) are used.

Figure 1c shows schematically the production of the luciferase enzyme,

- 15 Figure 2 shows the plasmid pTetLux1 (SEQ ID NO: 3).

Figure 3 shows the plasmid pTetLuc1 (SEQ ID NO: 1).

- Figure 4a shows the production of light (induction factor) versus concentration of tetracycline in samples for three different tetracyclines,
- 20

Figure 4b shows the production of light (induction factor) versus concentration of tetracycline in samples for further four different tetracyclines.

- 25 Figure 5 shows the effect of magnesium ions on the sensitivity of the method according to the invention.

Figure 6 illustrates possibilities of changing the assay window for the method of the invention by adjusting magnesium ion concentration and pH.

Figure 7 shows the induction factor versus tetracycline concentration when using freeze-dried *E. coli* in the determination of tetracycline.

Figure 8 shows a comparison of the assays based on using cells with the plasmid pTetLuc1 (SEQ ID NO: 1) and with the plasmid pTetLux1 (SEQ ID NO: 3).

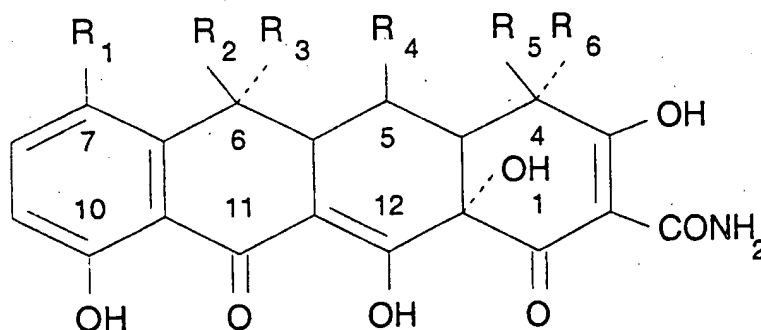
Figure 9 shows induction factors versus antibiotic concentrations of a pig serum sample (cells *E. coli* K12, pTetLux1).

Figure 10 shows the effect of EDTA in a milk sample assay, and

Figure 11 shows the light emission versus time for an assay according to the invention.

DETAILED DESCRIPTION OF THE INVENTION

The term "tetracycline" shall be understood to include any compound covered by the general structure formula



and particularly the specific commercially available compounds listed in the table below.

GENERIC NAME	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆
Chlorotetracycline	Cl	OH	CH ₃	H	H	N(CH ₃) ₂
Demethylchlorotetracycline	Cl	OH	H	H	H	N(CH ₃) ₂
Doxycycline	H	H	CH ₃	OH	H	N(CH ₃) ₂
Methacycline	H	CH ₃	H	OH	H	N(CH ₃) ₂
Minocycline	N(CH ₃) ₂	H	H	H	H	N(CH ₃) ₂
Oxytetracycline	H	OH	CH ₃	OH	H	N(CH ₃) ₂
Tetracycline	H	OH	CH ₃	H	H	N(CH ₃) ₂

Furthermore, the term "tetracycline" shall be understood to cover the metabolic and other reformulation/decomposition products thereof.

5

The cells useful in the method of the invention are preferably *Escherichia coli*, which are stored in dried form, e.g. in lyophilized form before their use in the method according to the invention. Also freshly cultivated cells can be used.

- 10 According to a preferred embodiment, the DNA vector including a nucleotide sequence encoding a light producing enzyme is a plasmid containing the luxCDABE genes (SEQ ID NO: 3), tetracycline repressor (TetR) (SEQ ID NO: 11) and tetracycline promotor (TetA) (SEQ ID NO: 9) from transposon *Tn10*. Particularly preferable is the plasmid pTetLux1 (SEQ ID NO: 3).

15

According to another preferred embodiment, the DNA vector including a nucleotide sequence encoding a light producing enzyme is a plasmid containing the insect

luciferase gene, tetracycline repressor (TetR) (SEQ ID NO: 11) and tetracycline promotor (TetA) (SEQ ID NO: 9) from *Tn10*. In this case the substrate for insect luciferase reaction, D-luciferin, is added to the mixture of the sample and the cells in order to initiate the luminescence of the cells. The plasmid is preferably pTetLuc1
5 (SEQ ID NO: 1).

The method according to this invention is useful for the determination of tetracycline in various kinds of samples. As examples can be mentioned milk, fish, meat, infant formula, eggs, honey, vegetables, serum, plasma, whole blood or the
10 like.

The luminescence of the cells is preferably measured using an X-ray or polaroid film, a CCD-camera (Charge Coupled Device), a liquid scintillation counter or, most preferably, a luminometer.

15

The sensitivity of this analysis method with respect to the tetracycline can be controlled by increasing or decreasing the concentration of divalent metal ions, e.g. magnesium ions, in the mixture of the sample and the cells, by adjusting the pH or by combined adjusting of the divalent metal ion concentration and the pH.

20 Increasing concentration of magnesium ions decreases the sensitivity and vice versa. Increasing pH will also cause a decreasing sensitivity. The sensitivity of the analysis with respect to the tetracycline can be increased by the use of cells which are especially antibiotic sensitive mutant strains. Chelating agents such as EDTA can be added to further sensitize the sensor system for tetracyclines.

25

Figures 1 show a schematic representation of a method based on specific detection of the presence of tetracyclines using microbial cells cloned with either the plasmid pTetLux1 (SEQ ID NO: 3) (Figure 1a) or with the plasmid pTetLuc1 (SEQ ID

NO: 1) (Figure 1b). The figures show that cells containing either of the plasmids can be triggered to produce light by adding a chemical agent (a tetracycline). Light production is a consequence of tetracycline responsive promoter activation due to removal of the tet-repressor protein (SEQ ID NO: 11) leading to the production of
5 luciferase specific mRNA and luciferase protein (SEQ ID NO: 2, 4-8) itself. The principle is demonstrated in Figure 1c. In case of the usage of full length bacterial luciferase operon (SEQ ID NO: 3) containing *luxC*, *luxD*, *luxA*, *luxB* and *luxE* genes (SEQ ID NO: 3) (Figure 1a), one is able to get light emission without addition of any substance. In case of insect (e.g. firefly) luciferase (SEQ ID NO: 2) (Figure
10 1b), light is emitted only after the addition of D-luciferin. It should be noticed that the triggering of luciferase synthesis and light production commences immediately when the cells are introduced to the inducer molecules (tetracyclines). Therefore there is no need to use dividing cells and hence there is no need to use long cultivation of microbial cells such as the case is with conventional methods.
15 Therefore, if needed, one can get results in minutes rather than in hours or days which is the case when conventional methods are used.

Figure 2a shows the plasmid pTetLux1 (SEQ ID NO: 3), in which the production of bacterial luciferase (SEQ ID NO: 4-8) of *Photorhabdus luminescens* (formerly
20 *Xenorhabdus luminescens*; the lux-operon structure and the full-length nucleotide sequence of *P. luminescens* was published in Szittner, R. and Meighen, E. (1990) J. Biol. Chem. 265, 16581-16587) can be switched on by the addition of a chemical agent belonging to the tetracycline family of antimicrobial agents in a cloned *E. coli* bacterium. SEQ ID NO: 3 shows the nucleotide sequence of the plasmid pTetLux1.
25 This plasmid construct is devised to contain the five genes from *P. luminescens* luciferase operon necessary for the light production without any additions of substrates, i.e. cells cloned with such a construct produce substrates endogenously. By incubating *E. coli* cells containing this plasmid (or any other microbial strain

wherein a similar regulation/reporter gene system is incorporated containing the necessary secondary regulatory sequences in the constructs such as correct ribosome binding region, transcriptional termination etc.) in the presence of very small amounts of tetracyclines one is able to obtain light production the intensity of which is proportional to the concentration of tetracycline used.

Any *E. coli* mutant strain and especially those strains having a mutation in the export/import machinery of the membranes or otherwise leaky character making it possible for large molecules to easily penetrate inside the cell would be beneficial to use in the method described in this invention. Also other gram-negative bacteria such as strains belonging to genus *Salmonella*, *Shigella*, *Enterobacter*, *Citrobacter*, *Klebsiella*, *Erwinia*, *Pseudomonas*, *Serratia* as well as gram-positive organisms such as those belonging to genus *Bacillus* (especially *B. subtilis*, *B. licheniformis*, *B. pumilus*, *B. globigii*, *B. natto*, *B. amyloliquefaciens* as well as *B. niger*, *B. brevis*, *B. megaterium*), *Streptomyces*, *Lactobacillus* (especially *L. lactis*, *L. casei*) and *Streptococcus* (especially *S. thermophilus*, *S. cremoris*, *S. agalactiae*) come into question. Especially asporogenic strains of *Bacilli* or *Lactobacilli* are suitable.

Figure 3 shows the plasmid pTetLuc1 (SEQ ID NO: 1), in which the production of firefly luciferase (SEQ ID NO: 2) of *Photinus pyralis* (The gene encoding firefly luciferase was originally cloned and sequenced in the middle of the 1980's by DeWet, J. et al. (1987) Mol. Cell. Biol. 7, 725-737) can be switched on by the addition of a chemical agent belonging to the tetracycline family of antimicrobial agents in a cloned *E. coli* bacterium. SEQ ID NO: 1 shows the nucleotide sequence of this plasmid. By incubating *E. coli* cells containing this plasmid (or any other microbial strain wherein a similar regulation/reporter gene system is incorporated containing the necessary secondary regulatory sequences in the constructs such as correct ribosome binding region, transcriptional termination etc.) in the presence of

very small amounts of tetracyclines one is able to obtain light production by the addition of D-luciferin, which is the substrate of firefly luciferase. The intensity of light emission is proportional to the concentration of tetracycline used.

- 5 Figures 4a and 4b shows the effect of altogether seven different tetracyclines on the production of light as a function of concentration of each tetracycline. As controls different non-tetracycline antibiotics were included in this study to show that the sensor strain is specific for the tetracyclines. The luminescence was emitted from *E. coli* containing the plasmid pTetLux1 (SEQ ID NO: 3). The detection was made
10 after an incubation of 90 min. All tetracyclines tested behaved in a very similar manner and induction efficiencies were at the same antibiotic concentration area. This makes this sensor even more attractive for analytical use for the determination of the tetracycline group of antibiotics.
- 15 It should be noted that the accumulation of various tetracyclines into microbial cells is very strongly affected by the extracellular concentration of Mg^{2+} ions. Figure 5 shows the effect of increasing concentrations of Mg^{2+} ions on the behavior of *E. coli* cells containing the plasmid pTetLux1 (SEQ ID NO: 3). As can be seen the tetracycline response curve is shifted to the right as a function of increasing
20 concentrations of added Mg^{2+} ions. Thus by increasing the Mg^{2+} ion concentration one is able to decrease the sensitivity of the tetracycline sensor described in this invention. This fact is of great importance in cases where one does not need a high sensitivity of the measurement and where the approximate concentration of the ion is roughly constant and known such as in milk, serum and plasma.
- 25 The sensitivity can be increased by removing magnesium ions from the assay mixture e.g. by adding a chelating agent forming a complex with magnesium.

Figure 6 shows the possibilities to change the assay window for tetracyclines by adjusting the magnesium ion concentration and by combined adjustment of the magnesium ion concentration and pH.

- 5 The sensitivity of the assay can be increased by the use of cells which are especially antibiotic sensitive mutant strains. Hundreds of specific mutations for bacteria are known with which it is possible to study the activity of specific reactions. For instance trace amounts of antibiotics cause visible changes in the metabolism or in the cell membranes of antibiotic sensitive bacterial mutants. Mutations in cell wall
- 10 structural components or biosynthetic enzymes as well as in transport and efflux proteins such as porins might have an effect on the behavior of each sensor. Using these kinds of mutations one is able to develop tests measuring residual antibiotics from biological material very sensitively. It is also rather simple to transfer new characteristics into bacterial cells by genetic engineering techniques. This
- 15 phenomenon broadens the applicability of these organisms in tests utilizing whole cell sensor.

Measurement of light emission can be done by using X-ray or polaroid film, using a liquid scintillation counter, a CCD-camera or a luminometer. The CCD-camera is an

20 instrument which is capable of detecting very low levels of light. In the applications of this invention such kind of a device could be used for the detection of tetracycline residues in food material such as vegetables or meat. The detection of light emission could be directly monitored from the surface of the food material sprayed with engineered luminescent bacteria. Either chemiluminescent (such as peroxidase -

25 luminol) or bioluminescent (such as luciferase - luciferin) reactions can be utilized. The luminometric method is performed with the aid of genes encoding either bacterial or beetle luciferases such as those described in the Figures 2 and 4. Several luminescent bacterial species such as *V. harveyi*, *V. fischeri*, *P. leiognathi*,

- P. phosphoreum*, *Xenorhabdus luminescens* etc. exist. Luminescent beetles are for example *Luciola mingrelica*, *Photinus pyralis*, *Pyrophorus plagiophthalmus*, *Lampyris noctiluca*, *Pholas dactylus*, etc. Also several eukaryotic species in the sea which luminesce, such as marine ostracod *Vargula hilgendorfii*, jellyfish *Aequorea victoria*, batrachoidid fish *Porichthys notatus*, pempherid fish *Parapriacanthus ransonneti* etc. exist. Fluorescent reporter proteins such as green fluorescent protein (GFP) or any of its variants could be used in the methods described in this invention (Li, X. et al. (1997) J. Biol. Chem. 272, 28545-28549).
- 10 In this invention high detection sensitivity of the luminescent enzyme labels inside a living cell associated with tetracycline-specific induction of label synthesis is based on the use of optimal concentration of all the reactants inside the cell including the necessary cofactors and accessory enzymes. All luciferase genes from these organisms would presumably work in a similar manner as the two examples shown
- 15 in this invention. These systems together with enhancers and modulators (wavelength, emission kinetics etc.) of light emission has been described in more detail in Campbell, A. "Chemiluminescence; principles and applications in biology and medicine", Weinheim; Deerfield Beach, Fl.; VCH; Chichester: Horwood, 1988.
- 20 Peroxidases or oxidases can be used together with compounds such as luminol or acridines (for instance lucigenin) to yield luminescent signals suitable for a detection system described here. Enzymatically generated chemiluminescence offers great sensitivity and rapid detection, too, in assays described in this invention. Thermally stable dioxetanes (such as AMPPD and Lumigen PPD) can be
- 25 enzymatically (such as alkaline phosphatase or β -galactosidase) triggered to produce chemiluminescence (Schaap, A.P. et al. (1989) Clin. Chem. 35, 1863-1864). The only difference to the luciferase enzymes would be that these enzymes are capable

of cleaving a man-made substrate which gives light emission (chemiluminescence) and the luciferases cleave natural substrates to produce light (bioluminescence).

- Tetracycline-controlled expression systems are developed to express heterologous proteins in procaryotic and eucaryotic cells for the purpose of production under a tight control of tet-regulatory system (Skerra, A. (1994) *Gene* 151, 131-135; Gossen, M. and Bujard, H. (1995) *US Patent* 5,464,758 ; Lutz, R. and Bujard, H. (1997) *Nucleic Acids Res.* 25, 1203-1210).
- 10 A method to study various tetracyclines and their mode of action was developed by Chopra et al. (Chopra, I. et al. (1990) *Antimicrob. Agents Chemother.* 34, 111-116) The assay system developed in this study was based on expression of β -galactosidase gene inserted under the control of tetA-gene. The method resulted in less sensitive detection of tetracyclines compared to the invention described here.
- 15 However in order to obtain maximum sensitivities Chopra et al. showed that it was necessary to add cyclic AMP (cAMP) to the medium which is an extremely expensive molecule to be used in routine applications. Furthermore, the method described by Chopra et al. contains a cell disruption stage by sonication in order to assay for the reporter gene activity, β -galactosidase, which step is not practical.
- 20 Instead, the method described in this invention does not contain any cell disruption. The activity of luciferase can be measured directly from living cells in real-time and in the case of pTetLux1 (SEQ ID NO: 3) there is no need of addition of any substrates. Therefore, promoter activation due to the presense/absense of tetracycline can be monitored continuously.

25

EXPERIMENTS

As cloning hosts and in antibiotic residue measurements various *E. coli* MC1061 (*ci+*, *araD139*, Δ (*ara-leu*)7696, *lacX74*, *galU*, *galK*, *hsr*, *hsm*, *strA*) (Casadaban,

M.J. and Cohen, S.N. (1980) J. Mol. Biol. 138, 179-207), BW322 (CGSC, *rfa210::Tn10, thi-1, relA1, spoT1, pyrE*) and K-12 (M72 Sm^R *lacZm-ΔbiouvR, trpEA2, Nam7Nam53cI857 HI*) (Remaut, E. et al. (1981) Gene 15, 81-93) can be used. Especially the strain LH530 (Hirvas, L. et al. (1997) Microbiology 143, 73-81) which has a decreased rate of lipid A biosynthesis. It has proven to be hypersusceptible to many different antibiotics.

Cells were grown on appropriate minimal agar-plates and were kept maximally one month at +4 °C after which new plates were streaked. The strains were kept also in 15% glycerol at -70 °C, where from growth was started through minimal plates. The cells were first cultivated in 5 ml of 2xTY medium (16 g Bacto tryptone, 8 g Yeast extract, 8 g NaCl, H₂O ad 1 l, pH 7.4, with appropriate antibiotic) 10 h at 30 °C in a shaker after which the cultivation was transferred to a bigger volume for 10 h with same medium.

15

Construction of tetracycline-responsive sensor plasmids:

To construct a recombinant DNA vector carrying luciferase genes under the control of a tetracycline responsive elements two new vectors were created. In the first one modified firefly luciferase gene (SEQ ID NO: 1) from vector pBLuc* (Bonin, A.L. et al. (1994) Gene 141, 75-77) was excised by using restriction enzymes *Xba*I and *Hin*DIII and the 1.7 kb fragment was isolated from LGT-agarose gel and purified using Qiagen gel extraction kit. This DNA-fragment containing the entire *Photinus pyralis* luciferase gene (SEQ ID NO: 1) was ligated using T4-DNA-ligase enzyme to vector pASK75 (Skerra, A. (1994) Gene 151, 131-135) which was previously restricted with the same restriction enzymes *Xba*I and *Hin*DIII and calf intestinal phosphatase treated to remove the protruding phosphate groups in order to prevent self-ligation. The resulting ligation mixture was incubated 3 hours at room temperature after which one μl of the mixture was electroporated according to

Dower *et al.* (Dower, W.J. *et al.* (1988) *Nucleic Acids Res.* 16, 6126-6144) into electrocompetent *E. coli* MC1061 cells. A plasmid was extracted from one of the colonies obtained and checked for the estimated structure by appropriate restriction enzyme digestions and agarose gel electrophoretic techniques. The plasmid obtained
5 was named as pTetLuc1 (SEQ ID NO: 1).

The plasmid containing the luxCDABE genes (SEQ ID NO: 3) of *Photobacterium luminescens* under the control of tetracycline responsive element was created as follows: Plasmid pASK75 was cut with restriction enzyme *EcoRI* and CIP-treated.
10 The linearized plasmid was separated on a LGT-agarose gel electrophoresis and the agarose was removed by using the Qiagen kit. The lux operon was excised with *EcoRI* from plasmid pCGLS-11 (Frackman, S. *et al.* (1990) *J. Bacteriol.* 172, 5767-5773), gel purified as above and ligated to pASK75 by using T4-DNA-ligase at 16 °C overnight. The ligation mixture was electroporated into *E. coli* MC1061 cells as
15 described above and correct transformants were screened for their ability to produce light (as measured with a BioOrbit 1250 manual luminometer) which production was increased in the presence of 1 µg/ml of tetracycline-HCl. The plasmid was further verified by restriction enzyme digestions and the correct structure was named as pTetLux1 (SEQ ID NO: 3). All the DNA-manipulations were performed
20 according to Sambrook *et al.*, "Molecular Cloning: A laboratory Manual, Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1989.

The vector pASK75 was utilized in the construction of tet-sensor plasmids shown in this invention. The vector pASK75 was originally developed for protein production
25 and purification purposes. It contains a signal sequence for secretion of the recombinant protein into the periplasmic space of *E. coli*. Also a C-terminal fusion between a purification tail, the Strept-tag, was incorporated into the vector to facilitate purification of recombinant protein using streptavidin affinity agarose gel

chromatography. The element controlling recombinant gene expression in the vector is tetA promoter/operator system that allows efficient regulation of the expression, which in Skerra's paper was described for the production and one-step purification of a murine single-chain antibody fragment. The tetA promoter/operator (SEQ ID NO: 9) is controlled by tetR-repressor (SEQ ID NO: 9) which is produced by the corresponding gene (SEQ ID NO: 9). Some of the above mentioned elements were eliminated from the present plasmids due to unnecessary features with respect to this invention.

10 **Transfer of the tetracycline sensor vectors to the antibiotic sensitive *E. coli* strain:**

Either pTetLux1 (SEQ ID NO: 3) or pTetLuc1 (SEQ ID NO: 1) was transformed into *E. coli* LH530 cells by electroporation as described above. The transformed cells were restreaked on agar plates and kept maximally for 2 weeks at +4 °C after which a new plate was streaked.

Use of the manipulated *E. coli* in tetracycline determination methods:

Example 1

Freeze-dried *E. coli* K-12/pTetLux1 were reconstituted with 1.0 ml of L-broth and bacteria were diluted 1:10 with 25 mM MES buffer in M9 minimal medium, pH 6.0. 190 µl bacterial suspension was added to microtiter plate wells containing 10 µl of tetracycline dilutions. The plate was incubated 90 minutes at 37 °C after which the plate was measured with Labsystems Luminoskan luminometer. As seen from Figure 7 the sensitivity of the assay of tetracycline is very high and comparable to that of fresh cells.

Example 2

Two different types of sensor DNA vector construct were compared. Strains *E. coli* K-12/pTetLux1 and *E. coli* K-12/pTetLuc1 were cultivated in L-broth media until optical density measured at 600 nm (OD600) was 1.5. The cells were diluted 1 to 50
5 with 25 mM MES-buffer in M9 minimal medium, pH 6.0 (Sambrook *et al.*, 1989, Cold Spring Harbor Laboratory, Cold Spring Harbor) and 190 µl was added to microtitration plate wells and 10 µl of sample dilution of tetracycline was added. After a 60 min incubation at 37 °C the light emission was measured using a Labsystems Luminoskan luminometer. Figure 8 shows the bioluminescence dose
10 response curve as a function of tetracycline added. As seen from the figure both systems (bacterial and insect luciferase) give roughly equal sensitivity of tetracycline detection.

One is able to use different luciferases instead of bacterial luciferase (SEQ ID
15 NO: 4-8) from *P. luminescens* without losing sensitivity or other performance of the test. Figure 8 shows an analogous measurement to the one in Figure 4b. In the plasmid used in this test (pTetLuc1) the bacterial luciferase was compensated with firefly luciferase (SEQ ID NO: 2) as described in Figure 3. The test was done essentially as with bacterial luciferase except that after the cells had been incubated
20 with or without tetracycline 10 minutes at 37 °C the cells were measured for light production after 15 minutes incubation time at 37 °C by adding 100 µl of solution containing 1 mM D-luciferin, in 0.1 M Na-citrate buffer, pH 5.0. Thereafter light production was measured using a manual luminometer 1250 (LKB-Wallac, Turku, Finland). As can be seen from Figure 8 sensitivity of the method to detect
25 tetracycline hydrochloride is extremely high and comparable to the detection made with bacterial luciferase.

Example 3

A lipemic pig serum was spiked at different concentrations of tetracycline, chlorotetracycline and oxytetracycline. Fresh *E. coli* K-12/pTetLux1 were diluted 1:50 with 25 mM MES buffer in M9 minimal medium, pH 6.0. 100 µl bacterial suspension was added to microtiter plate wells containing 100 µl of pig serum spiked with different tetracyclines. The plate was incubated 90 minutes at 37 °C after which the plate was measured with Labsystems Luminoskan luminometer. As seen from Figure 9 the sensitivity of the assay of different tetracyclines in pig serum matrix is very high.

Example 4

Tetracyclines will form chelate complexes with Ca^{2+} and Mg^{2+} in samples (e.g. milk), and lose their antimicrobial and induction activity in our assay system. Tetracyclines can be displaced from cation chelates by using strong chelating agents such as EDTA. Figure 10 shows the determination of tetracycline from a milk sample, which is spiked with different concentrations of tetracycline. Different amounts of EDTA were added to milk samples and this kind of displacement of cation-tetracycline complex clearly improved the sensitivity of the assay. In the assay we used freeze-dried *E. coli* K12/pTetLux1 that were reconstituted with L-broth 10 minutes in room temperature before the assay.

Example 5

Figure 11 shows the kinetics of bacterial bioluminescence after exposure of *E. coli* K-12/pTetLux1 to different dilutions of tetracycline antibiotics. The specific induction of tetracycline is very fast and specific light emission is seen already at the 10 minutes measuring point in the assay.

It will be appreciated that the methods of the present invention can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. It will be apparent for the specialist in the field that other embodiments exist and do not depart from the spirit of the invention. Thus, the described embodiments are

5 illustrative and should not be construed as restrictive.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: KORPELA, Matti
(B) STREET: Maijamaentie 13
(C) CITY: Naantali
(E) COUNTRY: Finland
(F) POSTAL CODE (ZIP): FIN-21100

(A) NAME: KARP, Matti
(B) STREET: Kampakatu 1
(C) CITY: Kaarina
(E) COUNTRY: Finland
(F) POSTAL CODE (ZIP): FIN-20660

(A) NAME: KURITTU, Jussi
(B) STREET: Puutarhakatu 16 A 20
(C) CITY: Turku
(E) COUNTRY: Finland
(F) POSTAL CODE (ZIP): FIN-20100

(ii) TITLE OF INVENTION: A NEW ASSAY METHOD

(iii) NUMBER OF SEQUENCES: 11

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: FI 974235
(B) FILING DATE: 14-NOV-1997

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 4846 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Photinus pyralis*

(vii) IMMEDIATE SOURCE:

(B) CLONE: pTetLuc1

(viii) POSITION IN GENOME:

(A) CHROMOSOME/SEGMENT: Plasmid

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION:1..3098
- (D) OTHER INFORMATION:/standard_name= "Vector pASK75"
/note= "Part of plasmid originating from vector pASK75;
feature description below, SEQ ID 9-11."
/citation= ([2])

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION:3119..4768
- (D) OTHER INFORMATION:/product= "Photinus pyralis
luciferase"
/citation= ([1])

(x) PUBLICATION INFORMATION:

- (A) AUTHORS: Bonin,
- (B) TITLE: Photinus pyralis luciferase: vectors that
contain a modified luc coding sequence allowing
convenient transfer into other systems
- (C) JOURNAL: Gene
- (D) VOLUME: 141
- (F) PAGES: 75-77
- (G) DATE: 1994
- (K) RELEVANT RESIDUES IN SEQ ID NO: 1: FROM 3099 TO 4772

(x) PUBLICATION INFORMATION:

- (A) AUTHORS: Skerra, A
- (B) TITLE: Use of the tetracycline promoter for the
tightly regulated production of a murine antibody
fragment in Escherichia coli
- (C) JOURNAL: Gene
- (D) VOLUME: 151
- (E) ISSUE: 1-2
- (F) PAGES: 131-135
- (G) DATE: 30-DEC-1994
- (K) RELEVANT RESIDUES IN SEQ ID NO: 1: FROM 1 TO 3098

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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(2) INFORMATION FOR SEQ ID NO: 2:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 550 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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Val Asn Ile Thr Tyr Ala Glu Tyr Phe Glu Met Ser Val Arg Leu Ala
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Glu Ala Met Lys Arg Tyr Gly Leu Asn Thr Asn His Arg Ile Val Val
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Cys Ser Glu Asn Ser Leu Gln Phe Phe Met Pro Val Leu Gly Ala Leu
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(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10220 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Photorhabdus luminescens

(vii) IMMEDIATE SOURCE:

- (B) CLONE: pTetLux1

(ix) FEATURE:

- (A) NAME/KEY: misc_feature
- (B) LOCATION: join(1..3190, 10140..10220)
- (D) OTHER INFORMATION: /standard_name= "vector pASK75"
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feature description below, SEQ ID NO: 9-11."
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(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 3634..5082
- (D) OTHER INFORMATION: /product= "Lux C"
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- (A) NAME/KEY: CDS
- (B) LOCATION: 5097..6017
- (D) OTHER INFORMATION: /product= "Lux D"
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- (A) NAME/KEY: CDS
- (B) LOCATION: 6069..7148
- (D) OTHER INFORMATION: /product= "Lux A"
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- (A) NAME/KEY: CDS
- (B) LOCATION: 7166..8146
- (D) OTHER INFORMATION: /product= "Lux B"
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(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 8256..9437
- (D) OTHER INFORMATION: /product= "Lux E"
/citation= ([1])

(x) PUBLICATION INFORMATION:

- (A) AUTHORS: Frackman,
- (B) TITLE: Cloning, organization and expression of the
bioluminescence genes of Xenorhabdus
luminescens
- (C) JOURNAL: J. Bacteriol.
- (D) VOLUME: 172
- (F) PAGES: 5767-5773
- (G) DATE: 1990
- (K) RELEVANT RESIDUES IN SEQ ID NO: 3: FROM 3191 TO 10139

(x) PUBLICATION INFORMATION:

- (A) AUTHORS: Skerra, A
 (B) TITLE: Use of the tetracycline promoter for the tightly regulated production of a murine antibody fragment in Escherichia coli
 (C) JOURNAL: Gene
 (D) VOLUME: 151
 (E) ISSUE: 1-2
 (F) PAGES: 131-135
 (G) DATE: 30-DEC-1994
 (K) RELEVANT RESIDUES IN SEQ ID NO: 3: FROM 1 TO 3190

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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GGGCTCCCTT TAGGGTTCCG ATTTAGTGCT TTACGGCACC TCGACCCCAA AAAACTTGAT	300
TAGGGTGATG GTTCACGTAG TGGGCCATCG CCCTGATAGA CGGTTTTTCG CCCTTTGACG	360
TTGGAGTCCA CGTTCTTTAA TAGTGACTC TTGTTCCAAA CTGGAACAAC ACTCAACCCT	420
ATCTCGGTCT ATTCTTTTGA TTTATAAGGG ATTTTGCCGA TTTCGGCCTA TTGGTTAAAA	480
AATGAGCTGA TTTAACAAAA ATTTAACGCG AATTTTAACA AAATATTAAC GCTTACAATT	540
TCAGGTGGCA CTTTTCGGGG AAATGTGCGC GGAACCCCTA TTTGTTTATT TTTCTAAATA	600
CATTCAAATA TGTATCCGCT CATGAGACAA TAACCCTGAT AAATGCTTCA ATAATATTGA	660
AAAAGGAAGA GTATGAGTAT TCAACATTTT CGTGTGCGCC TTATTCCTT TTTTGGCGCA	720
TTTTGCCTTC CTGTTTTTGC TCACCCAGAA ACGCTGGTGA AAGTAAAAGA TGCTGAAGAT	780
CAGTTGGGTG CACGAGTGGG TTACATCGAA CTGGATCTCA ACAGCGGTAA GATCCTTGAG	840
AGTTTTCGCC CCGAAGAACG TTTTCCAATG ATGAGCACTT TTAAAGTTCT GCTATGTGGC	900
GCGGTATTAT CCCGTATTGA CGCCGGGCAA GAGCAACTCG GTCGCCGCAT AACTATTCT	960
CAGAATGACT TGGTTGAGTA CTCACCAGTC ACAGAAAAGC ATCTTACGGA TGGCATGACA	1020
GTAAGAGAAT TATGCAGTGC TGCCATAACC ATGAGTGATA AACTGCGGC CACTTACTT	1080
CTGACAACGA TCGGAGGACC GAAGGAGCTA ACCGCTTTTT TGCACAACAT GGGGGATCAT	1140
GTAACTCGCC TTGATCGTTG GGAACCGGAG CTGAATGAAG CCATACCAA CGACGAGCGT	1200
GACACCACGA TGCCTGTAGC AATGGCAACA ACGTTGCGCA AACTATTAAC TGGCGAACTA	1260
CTTACTCTAG CTTCCTGGCA ACAATTGATA GACTGGATGG AGGCGGATAA AGTTGCAGGA	1320
CCACTTCTGC GCTCGGCCCT TCCGGCTGGC TGGTTTATTG CTGATAAATC TGGAGCCGGT	1380
GAGCGTGGCT CTCGCGGTAT CATTGCAGCA CTGGGGCCAG ATGGTAAGCC CTCCCGTATC	1440
GTAGTTATCT ACACGACGGG GAGTCAGGCA ACTATGGATG AACGAAATAG ACAGATCGCT	1500
GAGATAGGTG CCTCACTGAT TAAGCATTGG TAGGAATTAA TGATGTCTCG TTTAGATAAA	1560

AGTAAAGTGA TTAACAGCGC ATTAGAGCTG CTTAATGAGG TCGGAATCGA AGGTTTAAACA	1620
ACCCGTAAAC TCGCCCAGAA GCTAGGTGTA GAGCAGCCTA CATTGTATTG GCATGTAAAA	1680
AATAAGCGGG CTTTGCTCGA CGCCTTAGCC ATTGAGATGT TAGATAGGCA CCATACTCAC	1740
TTTTGCCCTT TAGAAGGGGA AAGCTGGCAA GATTTTTTAC GTAATAACGC TAAAAGTTTT	1800
AGATGTGCTT TACTAAGTCA TCGCGATGGA GCAAAAGTAC ATTTAGGTAC ACGGCCTACA	1860
GAAAAACAGT ATGAAACTCT CGAAAATCAA TTAGCCTTTT TATGCCAACA AGGTTTTTCA	1920
CTAGAGAATG CATTATATGC ACTCAGCGCA GTGGGGCATT TTACTTTAGG TTGCGTATTG	1980
GAAGATCAAG AGCATCAAGT CGCTAAAGAA GAAAGGGAAA CACCTACTAC TGATAGTATG	2040
CCGCCATTAT TACGACAAGC TATCGAATTA TTTGATCACC AAGGTGCAGA GCCAGCCTTC	2100
TTATTCGGCC TTGAATTGAT CATATGCGGA TTAGAAAAAC AACTTAAATG TGAAAGTGGG	2160
TCTTAAAAGC AGCATAACCT TTTTCCGTGA TGGTAACTTC ACTAGTTTAA AAGGATCTAG	2220
GTGAAGATCC TTTTGATAA TCTCATGACC AAAATCCCTT AACGTGAGTT TTCGTTCCAC	2280
TGAGCGTCAG ACCCCGTAGA AAAGATCAAA GGATCTTCTT GAGATCCTTT TTTTCTGCGC	2340
GTAATCTGCT GCTTGCAAAC AAAAAACCA CCGCTACCAG CGGTGGTTTG TTTGCCGGAT	2400
CAAGAGCTAC CAACTCTTTT TCCGAAGGTA ACTGGCTTCA GCAGAGCGCA GATACCAAAT	2460
ACTGTCTTTC TAGTGTAGCC GTAGTTAGGC CACCACTTCA AGAACTCTGT AGCACCGCCT	2520
ACATACCTCG CTCTGCTAAT CCTGTTACCA GTGGCTGCTG CCAGTGGCGA TAAGTCGTGT	2580
CTTACCGGGT TGGACTCAAG ACGATAGTTA CCGGATAAGG CGCAGCGGTC GGGCTGAACG	2640
GGGGGTTCGT GCACACAGCC CAGCTTGGAG CGAACGACCT ACACCGAACT GAGATACCTA	2700
CAGCGTGAGC TATGAGAAAG CGCCACGCTT CCCGAAGGGA GAAAGGCGGA CAGGTATCCG	2760
GTAAGCGGCA GGGTCGGAAC AGGAGAGCGC ACGAGGGAGC TTCCAGGGGG AAACGCCTGG	2820
TATCTTTATA GTCCTGTCGG GTTTCGCCAC CTCTGACTTG AGCGTCGATT TTTGTGATGC	2880
TCGTACGGGG GCGGAGCCT ATGGAAAAAC GCCAGCAACG CGGCCTTTTT ACGGTTCTCTG	2940
GCCTTTTGCT GGCCTTTTGC TCACATGACC CGACACCATC GAATGGCCAG ATGATTAAAT	3000
CCTAATTTTT GTTGACACTC TATCATTGAT AGAGTTATTT TACCACTCCC TATCAGTGAT	3060
AGAGAAAAGT GAAATGAATA GTTCGACAAA AATCTAGATA ACGAGGGCAA AAAATGAAAA	3120
AGACAGCTAT CGCGATTGCA GTGGCACTGG CTGGTTTCGC TACCGTAGCG CAGGCCTGAG	3180
ACCAGAATTC TTCTTTAGAA ATCTGCCGGT AAAAATTAGA TTGCTATTCA ATCTATTTCT	3240
ATCGGTATTT GTGAAATAAT ACTCAGGATA ATAATTTACA TAAATATTAT CACGCATTAG	3300
AGAAGAGCAT GACTTTTTTA ATTTAACTT TTCATTAACA AATCTTGTTG ATATGAAAAT	3360
TTTCCTTTGC TATTTTAAACA GATATTAAAA CGGGAATAGG CGTTATATTG ACGATCCATT	3420
CAGTTAGATT AAAAACCTTG AGCAGAAAAT TTATATTATT ATCATAATTA TGACGAAAGT	3480
TACAGGCCAG GAACCACGTA GTCAGAATCT GATTTTCTAT ATATTTGTTA TTTACATCGT	3540
CATAACACAA AAATATAAGA AGCAAGTGTT GGTACGACCA GTTCGCAAGA TAGTTAAACA	3600

GCAACTTAAG TTGAAATTAC CCCCATTAATA TGG ATG GCA AAT ATG ACT AAA AAA															3654	
Met Ala Asn Met Thr Lys Lys																
555																
ATT	TCA	TTC	ATT	ATT	AAC	GGC	CAG	GTT	GAA	ATC	TTT	CCC	GAA	AGT	GAT	3702
Ile	Ser	Phe	Ile	Ile	Asn	Gly	Gln	Val	Glu	Ile	Phe	Pro	Glu	Ser	Asp	
560 565 570																
GAT	TTA	GTG	CAA	TCC	ATT	AAT	TTT	GGT	GAT	AAT	AGT	GTT	TAC	CTG	CCA	3750
Asp	Leu	Val	Gln	Ser	Ile	Asn	Phe	Gly	Asp	Asn	Ser	Val	Tyr	Leu	Pro	
575 580 585																
ATA	TTG	AAT	GAC	TCT	CAT	GTA	AAA	AAC	ATT	ATT	GAT	TGT	AAT	GGA	AAT	3798
Ile	Leu	Asn	Asp	Ser	His	Val	Lys	Asn	Ile	Ile	Asp	Cys	Asn	Gly	Asn	
590 595 600 605																
AAC	GAA	TTA	CGG	TTG	CAT	AAC	ATT	GTC	AAT	TTT	CTC	TAT	ACG	GTA	GGG	3846
Asn	Glu	Leu	Arg	Leu	His	Asn	Ile	Val	Asn	Phe	Leu	Tyr	Thr	Val	Gly	
610 615 620																
CAA	AGA	TGG	AAA	AAT	GAA	GAA	TAC	TCA	AGA	CGC	AGG	ACA	TAC	ATT	CGT	3894
Gln	Arg	Trp	Lys	Asn	Glu	Glu	Tyr	Ser	Arg	Arg	Arg	Thr	Tyr	Ile	Arg	
625 630 635																
GAC	TTA	AAA	AAA	TAT	ATG	GGA	TAT	TCA	GAA	GAA	ATG	GCT	AAG	CTA	GAG	3942
Asp	Leu	Lys	Lys	Tyr	Met	Gly	Tyr	Ser	Glu	Glu	Met	Ala	Lys	Leu	Glu	
640 645 650																
GCC	AAT	TGG	ATA	TCT	ATG	ATT	TTA	TGT	TCT	AAA	GGC	GGC	CTT	TAT	GAT	3990
Ala	Asn	Trp	Ile	Ser	Met	Ile	Leu	Cys	Ser	Lys	Gly	Gly	Leu	Tyr	Asp	
655 660 665																
GTT	GTA	GAA	AAT	GAA	CTT	GGT	TCT	CGC	CAT	ATC	ATG	GAT	GAA	TGG	CTA	4038
Val	Val	Glu	Asn	Glu	Leu	Gly	Ser	Arg	His	Ile	Met	Asp	Glu	Trp	Leu	
670 675 680 685																
CCT	CAG	GAT	GAA	AGT	TAT	GTT	CGG	GCT	TTT	CCG	AAA	GGT	AAA	TCT	GTA	4086
Pro	Gln	Asp	Glu	Ser	Tyr	Val	Arg	Ala	Phe	Pro	Lys	Gly	Lys	Ser	Val	
690 695 700																
CAT	CTG	TTG	GCA	GGT	AAT	GTT	CCA	TTA	TCT	GGG	ATC	ATG	TCT	ATA	TTA	4134
His	Leu	Leu	Ala	Gly	Asn	Val	Pro	Leu	Ser	Gly	Ile	Met	Ser	Ile	Leu	
705 710 715																
CGC	GCA	ATT	TTA	ACT	AAG	AAT	CAG	TGT	ATT	ATA	AAA	ACA	TCG	TCA	ACC	4182
Arg	Ala	Ile	Leu	Thr	Lys	Asn	Gln	Cys	Ile	Ile	Lys	Thr	Ser	Ser	Thr	
720 725 730																
GAT	CCT	TTT	ACC	GCT	AAT	GCA	TTA	GCG	TTA	AGT	TTT	ATT	GAT	GTA	GAC	4230
Asp	Pro	Phe	Thr	Ala	Asn	Ala	Leu	Ala	Leu	Ser	Phe	Ile	Asp	Val	Asp	
735 740 745																
CCT	AAT	CAT	CCG	ATA	ACG	CGC	TCT	TTA	TCT	GTT	ATA	TAT	TGG	CCC	CAC	4278
Pro	Asn	His	Pro	Ile	Thr	Arg	Ser	Leu	Ser	Val	Ile	Tyr	Trp	Pro	His	
750 755 760 765																
CAA	GGT	GAT	ACA	TCA	CTC	GCA	AAA	GAA	ATT	ATG	CGA	CAT	GCG	GAT	GTT	4326
Gln	Gly	Asp	Thr	Ser	Leu	Ala	Lys	Glu	Ile	Met	Arg	His	Ala	Asp	Val	
770 775 780																
ATT	GTC	GCT	TGG	GGA	GGG	CCA	GAT	GCG	ATT	AAT	TGG	GCG	GTA	GAG	CAT	4374
Ile	Val	Ala	Trp	Gly	Gly	Pro	Asp	Ala	Ile	Asn	Trp	Ala	Val	Glu	His	
785 790 795																
GCG	CCA	TCT	TAT	GCT	GAT	GTG	ATT	AAA	TTT	GGT	TCT	AAA	AAG	AGT	CTT	4422
Ala	Pro	Ser	Tyr	Ala	Asp	Val	Ile	Lys	Phe	Gly	Ser	Lys	Lys	Ser	Leu	
800 805 810																

TGC ATT ATC GAT AAT CCT GTT GAT TTG ACG TCC GCA GCG ACA GGT GCG Cys Ile Ile Asp Asn Pro Val Asp Leu Thr Ser Ala Ala Thr Gly Ala 815 820 825	4470
GCT CAT GAT GTT TGT TTT TAC GAT CAG CGA GCT TGT TTT TCT GCC CAA Ala His Asp Val Cys Phe Tyr Asp Gln Arg Ala Cys Phe Ser Ala Gln 830 835 840 845	4518
AAC ATA TAT TAC ATG GGA AAT CAT TAT GAG GAA TTT AAG TTA GCG TTG Asn Ile Tyr Tyr Met Gly Asn His Tyr Glu Glu Phe Lys Leu Ala Leu 850 855 860	4566
ATA GAA AAA CTT AAT CTA TAT GCG CAT ATA TTA CCG AAT GCC AAA AAA Ile Glu Lys Leu Asn Leu Tyr Ala His Ile Leu Pro Asn Ala Lys Lys 865 870 875	4614
GAT TTT GAT GAA AAG GCG GCC TAT TCT TTA GTT CAA AAA GAA AGC TTG Asp Phe Asp Glu Lys Ala Ala Tyr Ser Leu Val Gln Lys Glu Ser Leu 880 885 890	4662
TTT GCT GGA TTA AAA GTA GAG GTG GAT ATT CAT CAA CGT TGG ATG ATT Phe Ala Gly Leu Lys Val Glu Val Asp Ile His Gln Arg Trp Met Ile 895 900 905	4710
ATT GAG TCA AAT GCA GGT GTG GAA TTT AAT CAA CCA CTT GGC AGA TGT Ile Glu Ser Asn Ala Gly Val Glu Phe Asn Gln Pro Leu Gly Arg Cys 910 915 920 925	4758
GTG TAC CTT CAT CAC GTC GAT AAT ATT GAG CAA ATA TTG CCT TAT GTT Val Tyr Leu His His Val Asp Asn Ile Glu Gln Ile Leu Pro Tyr Val 930 935 940	4806
CAA AAA AAT AAG ACG CAA ACC ATA TCT ATT TTT CCT TGG GAG TCA TCA Gln Lys Asn Lys Thr Gln Thr Ile Ser Ile Phe Pro Trp Glu Ser Ser 945 950 955	4854
TTT AAA TAT CGA GAT GCG TTA GCA TTA AAA GGT GCG GAA AGG ATT GTA Phe Lys Tyr Arg Asp Ala Leu Ala Leu Lys Gly Ala Glu Arg Ile Val 960 965 970	4902
GAA GCA GGA ATG AAT AAC ATA TTT CGA GTT GGT GGA TCT CAT GAC GGA Glu Ala Gly Met Asn Asn Ile Phe Arg Val Gly Gly Ser His Asp Gly 975 980 985	4950
ATG AGA CCG TTG CAA CGA TTA GTG ACA TAT ATT TCT CAT GAA AGG CCA Met Arg Pro Leu Gln Arg Leu Val Thr Tyr Ile Ser His Glu Arg Pro 990 995 1000 1005	4998
TCT AAC TAT ACG GCT AAG GAT GTT GCG GTT GAA ATA GAA CAG ACT CGA Ser Asn Tyr Thr Ala Lys Asp Val Ala Val Glu Ile Glu Gln Thr Arg 1010 1015 1020	5046
TTC CTG GAA GAA GAT AAG TTC CTT GTA TTT GTC CCA TAATAGGTAA Phe Leu Glu Glu Asp Lys Phe Leu Val Phe Val Pro 1025 1030	5092
AAGT ATG GAA AAT GAA TCA AAA TAT AAA ACC ATC GAC CAC GTT ATT TGT Met Glu Asn Glu Ser Lys Tyr Lys Thr Ile Asp His Val Ile Cys 1 5 10 15	5141
GTT GAA GGA AAT AAA AAA ATT CAT GTT TGG GAA ACG CTG CCA GAA GAA Val Glu Gly Asn Lys Lys Ile His Val Trp Glu Thr Leu Pro Glu Glu 20 25 30	5189
AAC AGC CCA AAG AGA AAG AAT GCC ATT ATT ATT GCG TCT GGT TTT GCC Asn Ser Pro Lys Arg Lys Asn Ala Ile Ile Ile Ala Ser Gly Phe Ala 35 40 45	5237

CGC AGG ATG GAT CAT TTT GCT GGT CTG GCG GAA TAT TTA TCG CGG AAT Arg Arg Met Asp His Phe Ala Gly Leu Ala Glu Tyr Leu Ser Arg Asn 50 55 60	5285
GGA TTT CAT GTG ATC CGC TAT GAT TCG CTT CAC CAC GTT GGA TTG AGT Gly Phe His Val Ile Arg Tyr Asp Ser Leu His His Val Gly Leu Ser 65 70 75	5333
TCA GGG ACA ATT GAT GAA TTT ACA ATG TCT ATA GGA AAG CAG AGC TTG Ser Gly Thr Ile Asp Glu Phe Thr Met Ser Ile Gly Lys Gln Ser Leu 80 85 90 95	5381
TTA GCA GTG GTT GAT TGG TTA ACT ACA CGA AAA ATA AAT AAC TTC GGT Leu Ala Val Val Asp Trp Leu Thr Thr Arg Lys Ile Asn Asn Phe Gly 100 105 110	5429
ATG TTG GCT TCA AGC TTA TCT GCG CGG ATA GCT TAT GCA AGC CTA TCT Met Leu Ala Ser Ser Leu Ser Ala Arg Ile Ala Tyr Ala Ser Leu Ser 115 120 125	5477
GAA ATC AAT GCT TCG TTT TTA ATC ACC GCA GTC GGT GTT GTT AAC TTA Glu Ile Asn Ala Ser Phe Leu Ile Thr Ala Val Gly Val Val Asn Leu 130 135 140	5525
AGA TAT TCT CTT GAA AGA GCT TTA GGG TTT GAT TAT CTC AGT CTA CCC Arg Tyr Ser Leu Glu Arg Ala Leu Gly Phe Asp Tyr Leu Ser Leu Pro 145 150 155	5573
ATT AAT GAA TTG CCG GAT AAT CTA GAT TTT GAA GGC CAT AAA TTG GGT Ile Asn Glu Leu Pro Asp Asn Leu Asp Phe Glu Gly His Lys Leu Gly 160 165 170 175	5621
GCT GAA GTC TTT GCG AGA GAT TGT CTT GAT TTT GGT TGG GAA GAT TTA Ala Glu Val Phe Ala Arg Asp Cys Leu Asp Phe Gly Trp Glu Asp Leu 180 185 190	5669
GCT TCT ACA ATT AAT AAC ATG ATG TAT CTT GAT ATA CCG TTT ATT GCT Ala Ser Thr Ile Asn Asn Met Met Tyr Leu Asp Ile Pro Phe Ile Ala 195 200 205	5717
TTT ACT GCA AAT AAC GAT AAT TGG GTC AAG CAA GAT GAA GTT ATC ACA Phe Thr Ala Asn Asn Asp Asn Trp Val Lys Gln Asp Glu Val Ile Thr 210 215 220	5765
TTG TTA TCA AAT ATT CGT AGT AAT CGA TGC AAG ATA TAT TCT TTG TTA Leu Leu Ser Asn Ile Arg Ser Asn Arg Cys Lys Ile Tyr Ser Leu Leu 225 230 235	5813
GGA AGT TCG CAT GAC TTG AGT GAA AAT TTA GTG GTC CTG CGC AAT TTT Gly Ser Ser His Asp Leu Ser Glu Asn Leu Val Val Leu Arg Asn Phe 240 245 250 255	5861
TAT CAA TCG GTT ACG AAA GCC GCT ATC GCG ATG GAT AAT GAT CAT CTG Tyr Gln Ser Val Thr Lys Ala Ala Ile Ala Met Asp Asn Asp His Leu 260 265 270	5909
GAT ATT GAT GTT GAT ATT ACT GAA CCG TCA TTT GAA CAT TTA ACT ATT Asp Ile Asp Val Asp Ile Thr Glu Pro Ser Phe Glu His Leu Thr Ile 275 280 285	5957
GCG ACA GTC AAT GAA CGC CGA ATG AGA ATT GAG ATT GAA AAT CAA GCA Ala Thr Val Asn Glu Arg Arg Met Arg Ile Glu Ile Glu Asn Gln Ala 290 295 300	6005
ATT TCT CTG TCT TAAAATCTAT TGAGATATTC TATCACTCAA ATAGCAATAT Ile Ser Leu Ser 305	6057

AAGGACTCTC T	ATG	AAA	TTT	GGA	AAC	TTT	TTG	CTT	ACA	TAC	CAA	CCT	CCC	6107
	Met	Lys	Phe	Gly	Asn	Phe	Leu	Leu	Thr	Tyr	Gln	Pro	Pro	
	1				5						10			
CAA TTT TCT CAA ACA GAG GTA ATG AAA CGT TTG GTT AAA TTA GGT CGC														6155
Gln Phe Ser Gln Thr Glu Val Met Lys Arg Leu Val Lys Leu Gly Arg														
	15				20					25				
ATC TCT GAG GAG TGT GGT TTT GAT ACC GTA TGG TTA CTG GAG CAT CAT														6203
Ile Ser Glu Glu Cys Gly Phe Asp Thr Val Trp Leu Leu Glu His His														
	30				35				40				45	
TTC ACG GAG TTT GGT TTG CTT GGT AAC CCT TAT GTC GCT GCT GCA TAT														6251
Phe Thr Glu Phe Gly Leu Leu Gly Asn Pro Tyr Val Ala Ala Ala Tyr														
				50				55				60		
TTA CTT GGC GCG ACT AAA AAA TTG AAT GTA GGA ACT GCC GCT ATT GTT														6299
Leu Leu Gly Ala Thr Lys Lys Leu Asn Val Gly Thr Ala Ala Ile Val														
			65				70				75			
CTT CCC ACA GCC CAT CCA GTA CGC CAA CTT GAA GAT GTG AAT TTA TTG														6347
Leu Pro Thr Ala His Pro Val Arg Gln Leu Glu Asp Val Asn Leu Leu														
			80				85				90			
GAT CAA ATG TCA AAA GGA CGA TTT CGG TTT GGT ATT TGC CGA GGG CTT														6395
Asp Gln Met Ser Lys Gly Arg Phe Arg Phe Gly Ile Cys Arg Gly Leu														
	95				100				105					
TAC AAC AAG GAC TTT CGC GTA TTC GGC ACA GAT ATG AAT AAC AGT CGC														6443
Tyr Asn Lys Asp Phe Arg Val Phe Gly Thr Asp Met Asn Asn Ser Arg														
	110				115				120				125	
GCC TTA GCG GAA TGC TGG TAC GGG CTG ATA AAG AAT GGC ATG ACA GAG														6491
Ala Leu Ala Glu Cys Trp Tyr Gly Leu Ile Lys Asn Gly Met Thr Glu														
			130				135					140		
GGA TAT ATG GAA GCT GAT AAT GAA CAT ATC AAG TTC CAT AAG GTA AAA														6539
Gly Tyr Met Glu Ala Asp Asn Glu His Ile Lys Phe His Lys Val Lys														
			145				150				155			
GTA AAC CCC GCG GCG TAT AGC AGA GGT GGC GCA CCG GTT TAT GTG GTG														6587
Val Asn Pro Ala Ala Tyr Ser Arg Gly Gly Ala Pro Val Tyr Val Val														
			160				165				170			
GCT GAA TCA GCT TCG ACG ACT GAG TGG GCT GCT CAA TTT GGC CTA CCG														6635
Ala Glu Ser Ala Ser Thr Thr Glu Trp Ala Ala Gln Phe Gly Leu Pro														
	175					180				185				
ATG ATA TTA AGT TGG ATT ATA AAT ACT AAC GAA AAG AAA GCA CAA CTT														6683
Met Ile Leu Ser Trp Ile Ile Asn Thr Asn Glu Lys Lys Ala Gln Leu														
	190				195				200				205	
GAG CTT TAT AAT GAA GTG GCT CAA GAA TAT GGG CAC GAT ATT CAT AAT														6731
Glu Leu Tyr Asn Glu Val Ala Gln Glu Tyr Gly His Asp Ile His Asn														
			210				215					220		
ATC GAC CAT TGC TTA TCA TAT ATA ACA TCT GTA GAT CAT GAC TCA ATT														6779
Ile Asp His Cys Leu Ser Tyr Ile Thr Ser Val Asp His Asp Ser Ile														
			225				230				235			
AAA GCG AAA GAG ATT TGC CGG AAA TTT CTG GGG CAT TGG TAT GAT TCT														6827
Lys Ala Lys Glu Ile Cys Arg Lys Phe Leu Gly His Trp Tyr Asp Ser														
			240				245				250			
TAT GTG AAT GCT ACG ACT ATT TTT GAT GAT TCA GAC CAA ACA AGA GGT														6875
Tyr Val Asn Ala Thr Thr Ile Phe Asp Asp Ser Asp Gln Thr Arg Gly														
	255				260				265					

TAT GAT TTC AAT AAA GGG CAG TGG CGT GAC TTT GTA TTA AAA GGA CAT Tyr Asp Phe Asn Lys Gly Gln Trp Arg Asp Phe Val Leu Lys Gly His 270 275 280 285	6923
AAA GAT ACT AAT CGC CGT ATT GAT TAC AGT TAC GAA ATC AAT CCC GTG Lys Asp Thr Asn Arg Arg Ile Asp Tyr Ser Tyr Glu Ile Asn Pro Val 290 295 300	6971
GGA ACG CCG CAG GAA TGT ATT GAC ATA ATT CAA AAA GAC ATT GAT GCT Gly Thr Pro Gln Glu Cys Ile Asp Ile Ile Gln Lys Asp Ile Asp Ala 305 310 315	7019
ACA GGA ATA TCA AAT ATT TGT TGT GGA TTT GAA GCT AAT GGA ACA GTA Thr Gly Ile Ser Asn Ile Cys Cys Gly Phe Glu Ala Asn Gly Thr Val 320 325 330	7067
GAC GAA ATT ATT GCT TCC ATG AAG CTC TTC CAG TCT GAT GTC ATG CCA Asp Glu Ile Ile Ala Ser Met Lys Leu Phe Gln Ser Asp Val Met Pro 335 340 345	7115
TTT CTT AAA GAA AAA CAA CGT TCG CTA TTA TAT TAGCTAAGGA GAAAGAA Phe Leu Lys Glu Lys Gln Arg Ser Leu Leu Tyr 350 355 360	7165
ATG AAA TTT GGA TTG TTC TTC CTT AAC TTC ATC AAT TCA ACA ACT GTT Met Lys Phe Gly Leu Phe Phe Leu Asn Phe Ile Asn Ser Thr Thr Val 1 5 10 15	7213
CAA GAA CAA AGT ATA GTT CGC ATG CAG GAA ATA ACG GAG TAT GTT GAT Gln Glu Gln Ser Ile Val Arg Met Gln Glu Ile Thr Glu Tyr Val Asp 20 25 30	7261
AAG TTG AAT TTT GAA CAG ATT TTA GTG TAT GAA AAT CAT TTT TCA GAT Lys Leu Asn Phe Glu Gln Ile Leu Val Tyr Glu Asn His Phe Ser Asp 35 40 45	7309
AAT GGT GTT GTC GGC GCT CCT CTG ACT GTT TCT GGT TTT CTG CTC GGT Asn Gly Val Val Gly Ala Pro Leu Thr Val Ser Gly Phe Leu Leu Gly 50 55 60	7357
TTA ACA GAG AAA ATT AAA ATT GGT TCA TTA AAT CAC ATC ATT ACA ACT Leu Thr Glu Lys Ile Lys Ile Gly Ser Leu Asn His Ile Ile Thr Thr 65 70 75 80	7405
CAT CAT CCT GTC GCC ATA GCG GAG GAA GCT TGC TTA TTG GAT CAG TTA His His Pro Val Ala Ile Ala Glu Glu Ala Cys Leu Leu Asp Gln Leu 85 90 95	7453
AGT GAA GGG AGA TTT ATT TTA GGG TTT AGT GAT TGC GAA AAA AAA GAT Ser Glu Gly Arg Phe Ile Leu Gly Phe Ser Asp Cys Glu Lys Lys Asp 100 105 110	7501
GAA ATG CAT TTT TTT AAT CGC CCG GTT GAA TAT CAA CAG CAA CTA TTT Glu Met His Phe Phe Asn Arg Pro Val Glu Tyr Gln Gln Gln Leu Phe 115 120 125	7549
GAA GAG TGT TAT GAA ATC ATT AAC GAT GCT TTA ACA ACA GGC TAT TGT Glu Glu Cys Tyr Glu Ile Ile Asn Asp Ala Leu Thr Thr Gly Tyr Cys 130 135 140	7597
AAT CCA GAT AAC GAT TTT TAT AGC TTC CCT AAA ATA TCT GTA AAT CCC Asn Pro Asp Asn Asp Phe Tyr Ser Phe Pro Lys Ile Ser Val Asn Pro 145 150 155 160	7645
CAT GCT TAT ACG CCA GGC GGA CCT CGG AAA TAT GTA ACA GCA ACC AGT His Ala Tyr Thr Pro Gly Gly Pro Arg Lys Tyr Val Thr Ala Thr Ser 165 170 175	7693

CAT CAT ATT GTT GAG TGG GCG GCC AAA AAA GGT ATT CCT CTC ATC TTT His His Ile Val Glu Trp Ala Ala Lys Lys Gly Ile Pro Leu Ile Phe 180 185 190	7741
AAG TGG GAT GAT TCT AAT GAT GTT AGA TAT GAA TAT GCT GAA AGA TAT Lys Trp Asp Asp Ser Asn Asp Val Arg Tyr Glu Tyr Ala Glu Arg Tyr 195 200 205	7789
AAA GCC GTT GCG GAT AAA TAT GAC GTT GAC CTA TCA GAG ATA GAC CAT Lys Ala Val Ala Asp Lys Tyr Asp Val Asp Leu Ser Glu Ile Asp His 210 215 220	7837
CAG TTA ATG ATA TTA GTT AAC TAT AAC GAA GAT AGT AAT AAA GCT AAA Gln Leu Met Ile Leu Val Asn Tyr Asn Glu Asp Ser Asn Lys Ala Lys 225 230 235 240	7885
CAA GAG ACG CGT GCA TTT ATT AGT GAT TAT GTT CTT GAA ATG CAC CCT Gln Glu Thr Arg Ala Phe Ile Ser Asp Tyr Val Leu Glu Met His Pro 245 250 255	7933
AAT GAA AAT TTC GAA AAT AAA CTT GAA GAA ATA ATT GCA GAA AAC GCT Asn Glu Asn Phe Glu Asn Lys Leu Glu Glu Ile Ile Ala Glu Asn Ala 260 265 270	7981
GTC GGA AAT TAT ACG GAG TGT ATA ACT GCG GCT AAG TTG GCA ATT GAA Val Gly Asn Tyr Thr Glu Cys Ile Thr Ala Ala Lys Leu Ala Ile Glu 275 280 285	8029
AAG TGT GGT GCG AAA AGT GTA TTG CTG TCC TTT GAA CCA ATG AAT GAT Lys Cys Gly Ala Lys Ser Val Leu Leu Ser Phe Glu Pro Met Asn Asp 290 295 300	8077
TTG ATG AGC CAA AAA AAT GTA ATC AAT ATT GTT GAT GAT AAT ATT AAG Leu Met Ser Gln Lys Asn Val Ile Asn Ile Val Asp Asp Asn Ile Lys 305 310 315 320	8125
AAG TAC CAC ATG GAA TAT ACC TAATAGATTT CGAGTTGCAG CGAGGCGGCA Lys Tyr His Met Glu Tyr Thr 325	8176
AGTGAACGAA TCCCCAGGAG CATAGATAAC TATGTGACTG GGGTGAGTGA AAGCAGCCAA	8236
CAAAGCAGCA GCTTGAAAG ATG AAG GGT ATA AAA GAG TAT GAC AGC AGT GCT Met Lys Gly Ile Lys Glu Tyr Asp Ser Ser Ala 1 5 10	8288
GCC ATA CTT TCT AAT ATT ATC TTG AGG AGT AAA ACA GGT ATG ACT TCA Ala Ile Leu Ser Asn Ile Ile Leu Arg Ser Lys Thr Gly Met Thr Ser 15 20 25	8336
TAT GTT GAT AAA CAA GAA ATT ACA GCA AGC TCA GAA ATT GAT GAT TTG Tyr Val Asp Lys Gln Glu Ile Thr Ala Ser Ser Glu Ile Asp Asp Leu 30 35 40	8384
ATT TTT TCG AGC GAT CCA TTA GTG TGG TCT TAC GAC GAG CAG GAA AAA Ile Phe Ser Ser Asp Pro Leu Val Trp Ser Tyr Asp Glu Gln Glu Lys 45 50 55	8432
ATC AGA AAG AAA CTT GTG CTT GAT GCA TTT CGT AAT CAT TAT AAA CAT Ile Arg Lys Lys Leu Val Leu Asp Ala Phe Arg Asn His Tyr Lys His 60 65 70 75	8480
TGT CGA GAA TAT CGT CAC TAC TGT CAG GCA CAC AAA GTA GAT GAC AAT Cys Arg Glu Tyr Arg His Tyr Cys Gln Ala His Lys Val Asp Asp Asn 80 85 90	8528

ATT	ACG	GAA	ATT	GAT	GAC	ATA	CCT	GTA	TTC	CCA	ACA	TCG	GTT	TTT	AAG	8576
Ile	Thr	Glu	Ile	Asp	Asp	Ile	Pro	Val	Phe	Pro	Thr	Ser	Val	Phe	Lys	
			95					100					105			
TTT	ACT	CGC	TTA	TTA	ACT	TCT	CAG	GAA	AAC	GAG	ATT	GAA	AGT	TGG	TTT	8624
Phe	Thr	Arg	Leu	Leu	Thr	Ser	Gln	Glu	Asn	Glu	Ile	Glu	Ser	Trp	Phe	
		110					115					120				
ACC	AGT	AGC	GGC	ACG	AAT	GGT	TTA	AAA	AGT	CAG	GTG	GCG	CGT	GAC	AGA	8672
Thr	Ser	Ser	Gly	Thr	Asn	Gly	Leu	Lys	Ser	Gln	Val	Ala	Arg	Asp	Arg	
	125					130					135					
TTA	AGT	ATT	GAG	AGA	CTC	TTA	GGC	TCT	GTG	AGT	TAT	GCG	ATG	AAA	TAT	8720
Leu	Ser	Ile	Glu	Arg	Leu	Leu	Gly	Ser	Val	Ser	Tyr	Gly	Met	Lys	Tyr	
140					145					150					155	
GTT	GGT	AGT	TGG	TTT	GAT	CAT	CAA	ATA	GAA	TTA	GTC	AAT	TTG	GGA	CCA	8768
Val	Gly	Ser	Trp	Phe	Asp	His	Gln	Ile	Glu	Leu	Val	Asn	Leu	Gly	Pro	
				160					165					170		
GAT	AGA	TTT	AAT	GCT	CAT	AAT	ATT	TGG	TTT	AAA	TAT	GTT	ATG	AGT	TTG	8816
Asp	Arg	Phe	Asn	Ala	His	Asn	Ile	Trp	Phe	Lys	Tyr	Val	Met	Ser	Leu	
			175					180					185			
GTG	GAA	TTG	TTA	TAT	CCT	ACG	ACA	TTT	ACC	GTA	ACA	GAA	GAA	CGA	ATA	8864
Val	Glu	Leu	Leu	Tyr	Pro	Thr	Thr	Phe	Thr	Val	Thr	Glu	Glu	Arg	Ile	
		190					195					200				
GAT	TTT	GTT	AAA	ACA	TTG	AAT	AGT	CTT	GAA	CGA	ATA	AAA	AAT	CAA	GGG	8912
Asp	Phe	Val	Lys	Thr	Leu	Asn	Ser	Leu	Glu	Arg	Ile	Lys	Asn	Gln	Gly	
	205					210					215					
AAA	GAT	CTT	TGT	CTT	ATT	GGT	TCG	CCA	TAC	TTT	ATT	TAT	TTA	CTC	TGC	8960
Lys	Asp	Leu	Cys	Leu	Ile	Gly	Ser	Pro	Tyr	Phe	Ile	Tyr	Leu	Leu	Cys	
220					225					230					235	
CAT	TAT	ATG	AAA	GAT	AAA	AAA	ATC	TCA	TTT	TCT	GGA	GAT	AAA	AGC	CTT	9008
His	Tyr	Met	Lys	Asp	Lys	Lys	Ile	Ser	Phe	Ser	Gly	Asp	Lys	Ser	Leu	
				240					245					250		
TAT	ATC	ATA	ACC	GGA	GGC	GGC	TGG	AAA	AGT	TAC	GAA	AAA	GAA	TCT	CTG	9056
Tyr	Ile	Ile	Thr	Gly	Gly	Gly	Trp	Lys	Ser	Tyr	Glu	Lys	Glu	Ser	Leu	
			255					260					265			
AAA	CGT	GAT	GAT	TTC	AAT	CAT	CTT	TTA	TTT	GAT	ACT	TTC	AAT	CTC	AGT	9104
Lys	Arg	Asp	Asp	Phe	Asn	His	Leu	Leu	Phe	Asp	Thr	Phe	Asn	Leu	Ser	
		270				275						280				
GAT	ATT	AGT	CAG	ATC	CGA	GAT	ATA	TTT	AAT	CAA	GTT	GAA	CTC	AAC	ACT	9152
Asp	Ile	Ser	Gln	Ile	Arg	Asp	Ile	Phe	Asn	Gln	Val	Glu	Leu	Asn	Thr	
	285					290					295					
TGT	TTC	TTT	GAG	GAT	GAA	ATG	CAG	CGT	AAA	CAT	GTT	CCG	CCG	TGG	GTA	9200
Cys	Phe	Phe	Glu	Asp	Glu	Met	Gln	Arg	Lys	His	Val	Pro	Pro	Trp	Val	
300					305					310					315	
TAT	GCG	CGA	GCG	CTT	GAT	CCT	GAA	ACG	TTG	AAA	CCT	GTA	CCT	GAT	GGA	9248
Tyr	Ala	Arg	Ala	Leu	Asp	Pro	Glu	Thr	Leu	Lys	Pro	Val	Pro	Asp	Gly	
				320					325					330		
ACG	CCG	GGG	TTG	ATG	AGT	TAT	ATG	GAT	GCG	TCA	GCA	ACC	AGT	TAT	CCA	9296
Thr	Pro	Gly	Leu	Met	Ser	Tyr	Met	Asp	Ala	Ser	Ala	Thr	Ser	Tyr	Pro	
			335					340					345			
GCA	TTT	ATT	GTT	ACC	GAT	GAT	GTC	GGG	ATA	ATT	AGC	AGA	GAA	TAT	GGT	9344
Ala	Phe	Ile	Val	Thr	Asp	Asp	Val	Gly	Ile	Ile	Ser	Arg	Glu	Tyr	Gly	
		350					355					360				

AAG TAT CCC GGC GTG CTC GTT GAA ATT TTA CGT CGC GTC AAT ACG AGG 9392
 Lys Tyr Pro Gly Val Leu Val Glu Ile Leu Arg Arg Val Asn Thr Arg
 365 370 375
 ACG CAG AAA GGG TGT GCT TTA AGC TTA ACC GAA GCG TTT GAT AGT 9437
 Thr Gln Lys Gly Cys Ala Leu Ser Leu Thr Glu Ala Phe Asp Ser
 380 385 390
 TGATATCCTT TGCCTAATTG TAAGTGAAT GCTTGCGTTA TATAAATCTG AATGACATCT 9497
 ACACCTTTACA AAATTCTCCA AAACATCCAC ATTTGGGTAC TTGATAGAGG TTTATGGGGT 9557
 TGGCTTAACA TTGTTCTCAT TGTTATTATT GGCTCAAAGC AAAAGGAGAT AACATGAAAA 9617
 AATTGGCAGT TATGCTTGCA TTGGGAATGA TTAGCTTTGG TGCAATGGCA GTTGATGGGT 9677
 ATAAAGATGC AAAGTTTGGC ATGACAGAAG AAGAGTTTCT TTCGAAGAGG TTATGTGATT 9737
 TTGAAAAATT TGAGGGAGAT TCTCGAATAG AAGAAGTATC ACTTTATTCA TGTTCGTACT 9797
 TTTCTTTTGC TAACAAAAAG CGTGAAGCAA TGGCATTTTT TTAAATGGG AAATTTAAAA 9857
 GATTAGAGAT TAATATTGGC AGACTTGTGA AGCCAGTAAG CAAATCGTTA ACGAAAAAGT 9917
 ACGGAGATGG ATCATCGTAT CCATCAAAG AAGAATTTGA GAACGCGCTA AAATACAATG 9977
 GAACTATGTC TATAGGTTAT GATAATAATA CGGTATTAGT TGATATACAT ATAATATGTG 10037
 GCAAAGAAGG CATAGAAACC AGTCAACTGA TTTATACGAG TCCAGATGTT TATACGCTCC 10097
 CAGATTTTCGG AGAAAAATC CAGGAATTAA AGGGATTAAA GGAATTCGAG CTCGGTACCC 10157
 GGGGATCCCT CGAGGTCGAC CTGCAGGCAG CGCTTGCGGT CACCCGCACT TCGGTGGTTA 10217
 ATA 10220

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 483 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Met Ala Asn Met Thr Lys Lys Ile Ser Phe Ile Ile Asn Gly Gln Val
 1 5 10 15
 Glu Ile Phe Pro Glu Ser Asp Asp Leu Val Gln Ser Ile Asn Phe Gly
 20 25 30
 Asp Asn Ser Val Tyr Leu Pro Ile Leu Asn Asp Ser His Val Lys Asn
 35 40 45
 Ile Ile Asp Cys Asn Gly Asn Asn Glu Leu Arg Leu His Asn Ile Val
 50 55 60
 Asn Phe Leu Tyr Thr Val Gly Gln Arg Trp Lys Asn Glu Glu Tyr Ser
 65 70 75 80
 Arg Arg Arg Thr Tyr Ile Arg Asp Leu Lys Lys Tyr Met Gly Tyr Ser
 85 90 95
 Glu Glu Met Ala Lys Leu Glu Ala Asn Trp Ile Ser Met Ile Leu Cys
 100 105 110

Ser Lys Gly Gly Leu Tyr Asp Val Val Glu Asn Glu Leu Gly Ser Arg
 115 120 125
 His Ile Met Asp Glu Trp Leu Pro Gln Asp Glu Ser Tyr Val Arg Ala
 130 135 140
 Phe Pro Lys Gly Lys Ser Val His Leu Leu Ala Gly Asn Val Pro Leu
 145 150 155 160
 Ser Gly Ile Met Ser Ile Leu Arg Ala Ile Leu Thr Lys Asn Gln Cys
 165 170 175
 Ile Ile Lys Thr Ser Ser Thr Asp Pro Phe Thr Ala Asn Ala Leu Ala
 180 185 190
 Leu Ser Phe Ile Asp Val Asp Pro Asn His Pro Ile Thr Arg Ser Leu
 195 200 205
 Ser Val Ile Tyr Trp Pro His Gln Gly Asp Thr Ser Leu Ala Lys Glu
 210 215 220
 Ile Met Arg His Ala Asp Val Ile Val Ala Trp Gly Gly Pro Asp Ala
 225 230 235 240
 Ile Asn Trp Ala Val Glu His Ala Pro Ser Tyr Ala Asp Val Ile Lys
 245 250 255
 Phe Gly Ser Lys Lys Ser Leu Cys Ile Ile Asp Asn Pro Val Asp Leu
 260 265 270
 Thr Ser Ala Ala Thr Gly Ala Ala His Asp Val Cys Phe Tyr Asp Gln
 275 280 285
 Arg Ala Cys Phe Ser Ala Gln Asn Ile Tyr Tyr Met Gly Asn His Tyr
 290 295 300
 Glu Glu Phe Lys Leu Ala Leu Ile Glu Lys Leu Asn Leu Tyr Ala His
 305 310 315 320
 Ile Leu Pro Asn Ala Lys Lys Asp Phe Asp Glu Lys Ala Ala Tyr Ser
 325 330 335
 Leu Val Gln Lys Glu Ser Leu Phe Ala Gly Leu Lys Val Glu Val Asp
 340 345 350
 Ile His Gln Arg Trp Met Ile Ile Glu Ser Asn Ala Gly Val Glu Phe
 355 360 365
 Asn Gln Pro Leu Gly Arg Cys Val Tyr Leu His His Val Asp Asn Ile
 370 375 380
 Glu Gln Ile Leu Pro Tyr Val Gln Lys Asn Lys Thr Gln Thr Ile Ser
 385 390 395 400
 Ile Phe Pro Trp Glu Ser Ser Phe Lys Tyr Arg Asp Ala Leu Ala Leu
 405 410 415
 Lys Gly Ala Glu Arg Ile Val Glu Ala Gly Met Asn Asn Ile Phe Arg
 420 425 430
 Val Gly Gly Ser His Asp Gly Met Arg Pro Leu Gln Arg Leu Val Thr
 435 440 445
 Tyr Ile Ser His Glu Arg Pro Ser Asn Tyr Thr Ala Lys Asp Val Ala
 450 455 460
 Val Glu Ile Glu Gln Thr Arg Phe Leu Glu Glu Asp Lys Phe Leu Val
 465 470 475 480

Phe Val Pro

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 307 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

```

Met Glu Asn Glu Ser Lys Tyr Lys Thr Ile Asp His Val Ile Cys Val
 1              5              10              15
Glu Gly Asn Lys Lys Ile His Val Trp Glu Thr Leu Pro Glu Glu Asn
              20              25              30
Ser Pro Lys Arg Lys Asn Ala Ile Ile Ile Ala Ser Gly Phe Ala Arg
              35              40              45
Arg Met Asp His Phe Ala Gly Leu Ala Glu Tyr Leu Ser Arg Asn Gly
              50              55              60
Phe His Val Ile Arg Tyr Asp Ser Leu His His Val Gly Leu Ser Ser
              65              70              75              80
Gly Thr Ile Asp Glu Phe Thr Met Ser Ile Gly Lys Gln Ser Leu Leu
              85              90              95
Ala Val Val Asp Trp Leu Thr Thr Arg Lys Ile Asn Asn Phe Gly Met
              100             105             110
Leu Ala Ser Ser Leu Ser Ala Arg Ile Ala Tyr Ala Ser Leu Ser Glu
              115             120             125
Ile Asn Ala Ser Phe Leu Ile Thr Ala Val Gly Val Val Asn Leu Arg
              130             135             140
Tyr Ser Leu Glu Arg Ala Leu Gly Phe Asp Tyr Leu Ser Leu Pro Ile
              145             150             155             160
Asn Glu Leu Pro Asp Asn Leu Asp Phe Glu Gly His Lys Leu Gly Ala
              165             170             175
Glu Val Phe Ala Arg Asp Cys Leu Asp Phe Gly Trp Glu Asp Leu Ala
              180             185             190
Ser Thr Ile Asn Asn Met Met Tyr Leu Asp Ile Pro Phe Ile Ala Phe
              195             200             205
Thr Ala Asn Asn Asp Asn Trp Val Lys Gln Asp Glu Val Ile Thr Leu
              210             215             220
Leu Ser Asn Ile Arg Ser Asn Arg Cys Lys Ile Tyr Ser Leu Leu Gly
              225             230             235             240
Ser Ser His Asp Leu Ser Glu Asn Leu Val Val Leu Arg Asn Phe Tyr
              245             250             255
Gln Ser Val Thr Lys Ala Ala Ile Ala Met Asp Asn Asp His Leu Asp
              260             265             270
Ile Asp Val Asp Ile Thr Glu Pro Ser Phe Glu His Leu Thr Ile Ala
              275             280             285

```

Thr Val Asn Glu Arg Arg Met Arg Ile Glu Ile Glu Asn Gln Ala Ile
 290 295 300

Ser Leu Ser
 305

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 360 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Met Lys Phe Gly Asn Phe Leu Leu Thr Tyr Gln Pro Pro Gln Phe Ser
 1 5 10 15
 Gln Thr Glu Val Met Lys Arg Leu Val Lys Leu Gly Arg Ile Ser Glu
 20 25 30
 Glu Cys Gly Phe Asp Thr Val Trp Leu Leu Glu His His Phe Thr Glu
 35 40 45
 Phe Gly Leu Leu Gly Asn Pro Tyr Val Ala Ala Ala Tyr Leu Leu Gly
 50 55 60
 Ala Thr Lys Lys Leu Asn Val Gly Thr Ala Ala Ile Val Leu Pro Thr
 65 70 75 80
 Ala His Pro Val Arg Gln Leu Glu Asp Val Asn Leu Leu Asp Gln Met
 85 90 95
 Ser Lys Gly Arg Phe Arg Phe Gly Ile Cys Arg Gly Leu Tyr Asn Lys
 100 105 110
 Asp Phe Arg Val Phe Gly Thr Asp Met Asn Asn Ser Arg Ala Leu Ala
 115 120 125
 Glu Cys Trp Tyr Gly Leu Ile Lys Asn Gly Met Thr Glu Gly Tyr Met
 130 135 140
 Glu Ala Asp Asn Glu His Ile Lys Phe His Lys Val Lys Val Asn Pro
 145 150 155 160
 Ala Ala Tyr Ser Arg Gly Gly Ala Pro Val Tyr Val Val Ala Glu Ser
 165 170 175
 Ala Ser Thr Thr Glu Trp Ala Ala Gln Phe Gly Leu Pro Met Ile Leu
 180 185 190
 Ser Trp Ile Ile Asn Thr Asn Glu Lys Lys Ala Gln Leu Glu Leu Tyr
 195 200 205
 Asn Glu Val Ala Gln Glu Tyr Gly His Asp Ile His Asn Ile Asp His
 210 215 220
 Cys Leu Ser Tyr Ile Thr Ser Val Asp His Asp Ser Ile Lys Ala Lys
 225 230 235 240
 Glu Ile Cys Arg Lys Phe Leu Gly His Trp Tyr Asp Ser Tyr Val Asn
 245 250 255
 Ala Thr Thr Ile Phe Asp Asp Ser Asp Gln Thr Arg Gly Tyr Asp Phe
 260 265 270

Asn Lys Gly Gln Trp Arg Asp Phe Val Leu Lys Gly His Lys Asp Thr
 275 280 285

Asn Arg Arg Ile Asp Tyr Ser Tyr Glu Ile Asn Pro Val Gly Thr Pro
 290 295 300

Gln Glu Cys Ile Asp Ile Ile Gln Lys Asp Ile Asp Ala Thr Gly Ile
 305 310 315 320

Ser Asn Ile Cys Cys Gly Phe Glu Ala Asn Gly Thr Val Asp Glu Ile
 325 330 335

Ile Ala Ser Met Lys Leu Phe Gln Ser Asp Val Met Pro Phe Leu Lys
 340 345 350

Glu Lys Gln Arg Ser Leu Leu Tyr
 355 360

(2) INFORMATION FOR SEQ ID NO: 7:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 327 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Met Lys Phe Gly Leu Phe Phe Leu Asn Phe Ile Asn Ser Thr Thr Val
 1 5 10 15

Gln Glu Gln Ser Ile Val Arg Met Gln Glu Ile Thr Glu Tyr Val Asp
 20 25 30

Lys Leu Asn Phe Glu Gln Ile Leu Val Tyr Glu Asn His Phe Ser Asp
 35 40 45

Asn Gly Val Val Gly Ala Pro Leu Thr Val Ser Gly Phe Leu Leu Gly
 50 55 60

Leu Thr Glu Lys Ile Lys Ile Gly Ser Leu Asn His Ile Ile Thr Thr
 65 70 75 80

His His Pro Val Ala Ile Ala Glu Glu Ala Cys Leu Leu Asp Gln Leu
 85 90 95

Ser Glu Gly Arg Phe Ile Leu Gly Phe Ser Asp Cys Glu Lys Lys Asp
 100 105 110

Glu Met His Phe Phe Asn Arg Pro Val Glu Tyr Gln Gln Gln Leu Phe
 115 120 125

Glu Glu Cys Tyr Glu Ile Ile Asn Asp Ala Leu Thr Thr Gly Tyr Cys
 130 135 140

Asn Pro Asp Asn Asp Phe Tyr Ser Phe Pro Lys Ile Ser Val Asn Pro
 145 150 155 160

His Ala Tyr Thr Pro Gly Gly Pro Arg Lys Tyr Val Thr Ala Thr Ser
 165 170 175

His His Ile Val Glu Trp Ala Ala Lys Lys Gly Ile Pro Leu Ile Phe
 180 185 190

Lys Trp Asp Asp Ser Asn Asp Val Arg Tyr Glu Tyr Ala Glu Arg Tyr
 195 200 205

Lys Ala Val Ala Asp Lys Tyr Asp Val Asp Leu Ser Glu Ile Asp His
 210 215 220
 Gln Leu Met Ile Leu Val Asn Tyr Asn Glu Asp Ser Asn Lys Ala Lys
 225 230 235 240
 Gln Glu Thr Arg Ala Phe Ile Ser Asp Tyr Val Leu Glu Met His Pro
 245 250 255
 Asn Glu Asn Phe Glu Asn Lys Leu Glu Glu Ile Ile Ala Glu Asn Ala
 260 265 270
 Val Gly Asn Tyr Thr Glu Cys Ile Thr Ala Ala Lys Leu Ala Ile Glu
 275 280 285
 Lys Cys Gly Ala Lys Ser Val Leu Leu Ser Phe Glu Pro Met Asn Asp
 290 295 300
 Leu Met Ser Gln Lys Asn Val Ile Asn Ile Val Asp Asp Asn Ile Lys
 305 310 315 320
 Lys Tyr His Met Glu Tyr Thr
 325

(2) INFORMATION FOR SEQ ID NO: 8:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 394 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Met Lys Gly Ile Lys Glu Tyr Asp Ser Ser Ala Ala Ile Leu Ser Asn
 1 5 10 15
 Ile Ile Leu Arg Ser Lys Thr Gly Met Thr Ser Tyr Val Asp Lys Gln
 20 25 30
 Glu Ile Thr Ala Ser Ser Glu Ile Asp Asp Leu Ile Phe Ser Ser Asp
 35 40 45
 Pro Leu Val Trp Ser Tyr Asp Glu Gln Glu Lys Ile Arg Lys Lys Leu
 50 55 60
 Val Leu Asp Ala Phe Arg Asn His Tyr Lys His Cys Arg Glu Tyr Arg
 65 70 75 80
 His Tyr Cys Gln Ala His Lys Val Asp Asp Asn Ile Thr Glu Ile Asp
 85 90 95
 Asp Ile Pro Val Phe Pro Thr Ser Val Phe Lys Phe Thr Arg Leu Leu
 100 105 110
 Thr Ser Gln Glu Asn Glu Ile Glu Ser Trp Phe Thr Ser Ser Gly Thr
 115 120 125
 Asn Gly Leu Lys Ser Gln Val Ala Arg Asp Arg Leu Ser Ile Glu Arg
 130 135 140
 Leu Leu Gly Ser Val Ser Tyr Gly Met Lys Tyr Val Gly Ser Trp Phe
 145 150 155 160
 Asp His Gln Ile Glu Leu Val Asn Leu Gly Pro Asp Arg Phe Asn Ala
 165 170 175

His Asn Ile Trp Phe Lys Tyr Val Met Ser Leu Val Glu Leu Leu Tyr
 180 185 190
 Pro Thr Thr Phe Thr Val Thr Glu Glu Arg Ile Asp Phe Val Lys Thr
 195 200 205
 Leu Asn Ser Leu Glu Arg Ile Lys Asn Gln Gly Lys Asp Leu Cys Leu
 210 215 220
 Ile Gly Ser Pro Tyr Phe Ile Tyr Leu Leu Cys His Tyr Met Lys Asp
 225 230 235 240
 Lys Lys Ile Ser Phe Ser Gly Asp Lys Ser Leu Tyr Ile Ile Thr Gly
 245 250 255
 Gly Gly Trp Lys Ser Tyr Glu Lys Glu Ser Leu Lys Arg Asp Asp Phe
 260 265 270
 Asn His Leu Leu Phe Asp Thr Phe Asn Leu Ser Asp Ile Ser Gln Ile
 275 280 285
 Arg Asp Ile Phe Asn Gln Val Glu Leu Asn Thr Cys Phe Phe Glu Asp
 290 295 300
 Glu Met Gln Arg Lys His Val Pro Pro Trp Val Tyr Ala Arg Ala Leu
 305 310 315 320
 Asp Pro Glu Thr Leu Lys Pro Val Pro Asp Gly Thr Pro Gly Leu Met
 325 330 335
 Ser Tyr Met Asp Ala Ser Ala Thr Ser Tyr Pro Ala Phe Ile Val Thr
 340 345 350
 Asp Asp Val Gly Ile Ile Ser Arg Glu Tyr Gly Lys Tyr Pro Gly Val
 355 360 365
 Leu Val Glu Ile Leu Arg Arg Val Asn Thr Arg Thr Gln Lys Gly Cys
 370 375 380
 Ala Leu Ser Leu Thr Glu Ala Phe Asp Ser
 385 390

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3098 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: circular

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:

- (B) CLONE: pASK75

(viii) POSITION IN GENOME:

- (A) CHROMOSOME/SEGMENT: vector

(ix) FEATURE:

- (A) NAME/KEY: promoter
- (B) LOCATION: 542..672
- (D) OTHER INFORMATION: /function= "beta-la promoter"
/label= beta-la
/citation= ([1])

(ix) FEATURE:

(A) NAME/KEY: CDS
 (B) LOCATION: 673..1530
 (D) OTHER INFORMATION: /product= "beta-la"
 /citation= ([1])

(ix) FEATURE:

(A) NAME/KEY: CDS
 (B) LOCATION: 1543..2163
 (D) OTHER INFORMATION: /product= "tetR"
 /citation= ([1])

(ix) FEATURE:

(A) NAME/KEY: misc_feature
 (B) LOCATION: 2713..2950
 (D) OTHER INFORMATION: /function= "ORI"
 /label= ORI
 /citation= ([1])

(ix) FEATURE:

(A) NAME/KEY: promoter
 (B) LOCATION: 2976..3073
 (D) OTHER INFORMATION: /function= "p tetA promoter"
 /citation= ([1])

(x) PUBLICATION INFORMATION:

(A) AUTHORS: Skerra, A
 (B) TITLE: Use of the tetracycline promoter for the
 tightly regulated production of a murine antibody
 fragment in Escherichia coli
 (C) JOURNAL: Gene
 (D) VOLUME: 151
 (E) ISSUE: 1-2
 (F) PAGES: 131-135
 (G) DATE: 30-DEC-1994
 (K) RELEVANT RESIDUES IN SEQ ID NO: 9: FROM 1 TO 3098

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

AGCTTGACCT GTGAAGTGAA AAATGGCGCA CATTGTGCGA CATTTTTTTTT GTCTGCCGTT	60
TACCGCTACT GCGTCACGGA TCTCCACGCG CCCTGTAGCG GCGCATTAAAG CGCGGCGGGT	120
GTGGTGGTTA CGCGCAGCGT GACCGCTACA CTTGCCAGCG CCCTAGCGCC CGCTCCTTTC	180
GCTTCTTCC CTTCTTTTCT CGCCACGTTT GCGGCTTTC CCCGTCAAGC TCTAAATCGG	240
GGGCTCCCTT TAGGGTTCGG ATTTAGTGCT TTACGGCACC TCGACCCCAA AAAACTTGAT	300
TAGGGTGATG GTTCACGTAG TGGGCCATCG CCCTGATAGA CGGTTTTTCG CCCTTTGACG	360
TTGGAGTCCA CGTTCTTTAA TAGTGGACTC TTGTTCCAAA CTGGAACAAC ACTCAACCTT	420
ATCTCGGTCT ATTCTTTTGA TTTATAAGGG ATTTTGCCGA TTTCGGCCTA TTGGTTAAAA	480
AATGAGCTGA TTAAACAAAA ATTTAACGCG AATTTTAACA AAATATTAAC GCTTACAATT	540
TCAGGTGGCA CTTTTCGGGG AAATGTGCGC GGAACCCCTA TTTGTTTATT TTTCTAAATA	600
CATTCAAATA TGTATCCGCT CATGAGACAA TAACCCTGAT AAATGCTTCA ATAATATTGA	660
AAAAGGAAGA GT ATG AGT ATT CAÄ CAT TTC CGT GTC GCC CTT ATT CCC	708
Met Ser Ile Gln His Phe Arg Val Ala Leu Ile Pro	
395 400 405	

TTT TTT GCG GCA TTT TGC CTT CCT GTT TTT GCT CAC CCA GAA ACG CTG Phe Phe Ala Ala Phe Cys Leu Pro Val Phe Ala His Pro Glu Thr Leu 410 415 420	756
GTG AAA GTA AAA GAT GCT GAA GAT CAG TTG GGT GCA CGA GTG GGT TAC Val Lys Val Lys Asp Ala Glu Asp Gln Leu Gly Ala Arg Val Gly Tyr 425 430 435	804
ATC GAA CTG GAT CTC AAC AGC GGT AAG ATC CTT GAG AGT TTT CGC CCC Ile Glu Leu Asp Leu Asn Ser Gly Lys Ile Leu Glu Ser Phe Arg Pro 440 445 450	852
GAA GAA CGT TTT CCA ATG ATG AGC ACT TTT AAA GTT CTG CTA TGT GGC Glu Glu Arg Phe Pro Met Met Ser Thr Phe Lys Val Leu Leu Cys Gly 455 460 465 470	900
GCG GTA TTA TCC CGT ATT GAC GCC GGG CAA GAG CAA CTC GGT CGC CGC Ala Val Leu Ser Arg Ile Asp Ala Gly Gln Glu Gln Leu Gly Arg Arg 475 480 485	948
ATA CAC TAT TCT CAG AAT GAC TTG GTT GAG TAC TCA CCA GTC ACA GAA Ile His Tyr Ser Gln Asn Asp Leu Val Glu Tyr Ser Pro Val Thr Glu 490 495 500	996
AAG CAT CTT ACG GAT GGC ATG ACA GTA AGA GAA TTA TGC AGT GCT GCC Lys His Leu Thr Asp Gly Met Thr Val Arg Glu Leu Cys Ser Ala Ala 505 510 515	1044
ATA ACC ATG AGT GAT AAC ACT GCG GCC AAC TTA CTT CTG ACA ACG ATC Ile Thr Met Ser Asp Asn Thr Ala Ala Asn Leu Leu Thr Thr Ile 520 525 530	1092
GGA GGA CCG AAG GAG CTA ACC GCT TTT TTG CAC AAC ATG GGG GAT CAT Gly Gly Pro Lys Glu Leu Thr Ala Phe Leu His Asn Met Gly Asp His 535 540 545 550	1140
GTA ACT CGC CTT GAT CGT TGG GAA CCG GAG CTG AAT GAA GCC ATA CCA Val Thr Arg Leu Asp Arg Trp Glu Pro Glu Leu Asn Glu Ala Ile Pro 555 560 565	1188
AAC GAC GAG CGT GAC ACC ACG ATG CCT GTA GCA ATG GCA ACA ACG TTG Asn Asp Glu Arg Asp Thr Thr Met Pro Val Ala Met Ala Thr Thr Leu 570 575 580	1236
CGC AAA CTA TTA ACT GGC GAA CTA CTT ACT CTA GCT TCC CGG CAA CAA Arg Lys Leu Leu Thr Gly Glu Leu Leu Thr Leu Ala Ser Arg Gln Gln 585 590 595	1284
TTG ATA GAC TGG ATG GAG GCG GAT AAA GTT GCA GGA CCA CTT CTG CGC Leu Ile Asp Trp Met Glu Ala Asp Lys Val Ala Gly Pro Leu Leu Arg 600 605 610	1332
TCG GCC CTT CCG GCT GGC TGG TTT ATT GCT GAT AAA TCT GGA GCC GGT Ser Ala Leu Pro Ala Gly Trp Phe Ile Ala Asp Lys Ser Gly Ala Gly 615 620 625 630	1380
GAG CGT GGC TCT CGC GGT ATC ATT GCA GCA CTG GGG CCA GAT GGT AAG Glu Arg Gly Ser Arg Gly Ile Ile Ala Ala Leu Gly Pro Asp Gly Lys 635 640 645	1428
CCC TCC CGT ATC GTA GTT ATC TAC ACG ACG GGG AGT CAG GCA ACT ATG Pro Ser Arg Ile Val Val Ile Tyr Thr Thr Gly Ser Gln Ala Thr Met 650 655 660	1476
GAT GAA CGA AAT AGA CAG ATC GCT GAG ATA GGT GCC TCA CTG ATT AAG Asp Glu Arg Asn Arg Gln Ile Ala Glu Ile Gly Ala Ser Leu Ile Lys 665 670 675	1524

CAT TGG TAGGAATTAA TG ATG TCT CGT TTA GAT AAA AGT AAA GTG ATT His Trp 680	Met Ser Arg Leu Asp Lys Ser Lys Val Ile 1 5 10	1572
AAC AGC GCA TTA GAG CTG CTT AAT GAG GTC GGA ATC GAA GGT TTA ACA Asn Ser Ala Leu Glu Leu Leu Asn Glu Val Gly Ile Glu Gly Leu Thr 15 20 25		1620
ACC CGT AAA CTC GCC CAG AAG CTA GGT GTA GAG CAG CCT ACA TTG TAT Thr Arg Lys Leu Ala Gln Lys Leu Gly Val Glu Gln Pro Thr Leu Tyr 30 35 40		1668
TGG CAT GTA AAA AAT AAG CGG GCT TTG CTC GAC GCC TTA GCC ATT GAG Trp His Val Lys Asn Lys Arg Ala Leu Leu Asp Ala Leu Ala Ile Glu 45 50 55		1716
ATG TTA GAT AGG CAC CAT ACT CAC TTT TGC CCT TTA GAA GGG GAA AGC Met Leu Asp Arg His His Thr His Phe Cys Pro Leu Glu Gly Glu Ser 60 65 70		1764
TGG CAA GAT TTT TTA CGT AAT AAC GCT AAA AGT TTT AGA TGT GCT TTA Trp Gln Asp Phe Leu Arg Asn Asn Ala Lys Ser Phe Arg Cys Ala Leu 75 80 85 90		1812
CTA AGT CAT CGC GAT GGA GCA AAA GTA CAT TTA GGT ACA CGG CCT ACA Leu Ser His Arg Asp Gly Ala Lys Val His Leu Gly Thr Arg Pro Thr 95 100 105		1860
GAA AAA CAG TAT GAA ACT CTC GAA AAT CAA TTA GCC TTT TTA TGC CAA Glu Lys Gln Tyr Glu Thr Leu Glu Asn Gln Leu Ala Phe Leu Cys Gln 110 115 120		1908
CAA GGT TTT TCA CTA GAG AAT GCA TTA TAT GCA CTC AGC GCA GTG GGG Gln Gly Phe Ser Leu Glu Asn Ala Leu Tyr Ala Leu Ser Ala Val Gly 125 130 135		1956
CAT TTT ACT TTA GGT TGC GTA TTG GAA GAT CAA GAG CAT CAA GTC GCT His Phe Thr Leu Gly Cys Val Leu Glu Asp Gln Glu His Gln Val Ala 140 145 150		2004
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TTA TTC GGC CTT GAA TTG ATC ATA TGC GGA TTA GAA AAA CAA CTT AAA Leu Phe Gly Leu Glu Leu Ile Ile Cys Gly Leu Glu Lys Gln Leu Lys 190 195 200		2148
TGT GAA AGT GGG TCT TAAAAGCAGC ATAACCTTTT TCCGTGATGG TAACCTCACT Cys Glu Ser Gly Ser 205		2203
AGTTTAAAAG GATCTAGGTG AAGATCCTTT TTGATAATCT CATGACCAAA ATCCCTTAAC		2263
GTGAGTTTTC GTTCCACTGA GCGTCAGACC CCGTAGAAAA GATCAAAGGA TCTTCTTGAG		2323
ATCCTTTTTT TCTGCGCGTA ATCTGCTGCT TGCAAACAAA AAAACCACCG CTACCAGCGG		2383
TGGTTTGT TT GCCGGATCAA GAGCTACCAA CTCTTTTTTCC GAAGGTAACT GGCTTCAGCA		2443
GAGCGCAGAT ACCAAATACT GTCCTTCTAG TGTAGCCGTA GTTAGGCCAC CACTTCAAGA		2503
ACTCTGTAGC ACCGCCTACA TACCTCGCTC TGCTAATCCT GTTACCAGTG GCTGCTGCCA		2563

GTGGCGATAA GTCGTGTCTT ACCGGGTTGG ACTCAAGACG ATAGTTACCG GATAAGGCGC 2623
 AGCGGTCGGG CTGAACGGGG GGTTCGTGCA CACAGCCAG CTTGGAGCGA ACGACCTACA 2683
 CCGAACTGAG ATACCTACAG CGTGAGCTAT GAGAAAGCGC CACGCTTCCC GAAGGGAGAA 2743
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 CAGGGGGAAA CGCCTGGTAT CTTTATAGTC CTGTCGGGTT TCGCCACCTC TGAATTGAGC 2863
 GTCGATTTTT GTGATGCTCG TCAGGGGGGC GGAGCCTATG GAAAAACGCC AGCAACGCGG 2923
 CCTTTTTACG GTTCCTGGCC TTTTGCTGGC CTTTTGCTCA CATGACCCGA CACCATCGAA 2983
 TGGCCAGATG ATTAATTCCT AATTTTGTGTT GACACTCTAT CATTGATAGA GTTATTTTAC 3043
 CACTCCCTAT CAGTGATAGA GAAAAGTGAA ATGAATAGTT CGACAAAAAT CTAGA 3098

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 286 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Met Ser Ile Gln His Phe Arg Val Ala Leu Ile Pro Phe Phe Ala Ala
 1 5 10 15
 Phe Cys Leu Pro Val Phe Ala His Pro Glu Thr Leu Val Lys Val Lys
 20 25 30
 Asp Ala Glu Asp Gln Leu Gly Ala Arg Val Gly Tyr Ile Glu Leu Asp
 35 40 45
 Leu Asn Ser Gly Lys Ile Leu Glu Ser Phe Arg Pro Glu Glu Arg Phe
 50 55 60
 Pro Met Met Ser Thr Phe Lys Val Leu Leu Cys Gly Ala Val Leu Ser
 65 70 75 80
 Arg Ile Asp Ala Gly Gln Glu Gln Leu Gly Arg Arg Ile His Tyr Ser
 85 90 95
 Gln Asn Asp Leu Val Glu Tyr Ser Pro Val Thr Glu Lys His Leu Thr
 100 105 110
 Asp Gly Met Thr Val Arg Glu Leu Cys Ser Ala Ala Ile Thr Met Ser
 115 120 125
 Asp Asn Thr Ala Ala Asn Leu Leu Leu Thr Thr Ile Gly Gly Pro Lys
 130 135 140
 Glu Leu Thr Ala Phe Leu His Asn Met Gly Asp His Val Thr Arg Leu
 145 150 155 160
 Asp Arg Trp Glu Pro Glu Leu Asn Glu Ala Ile Pro Asn Asp Glu Arg
 165 170 175
 Asp Thr Thr Met Pro Val Ala Met Ala Thr Thr Leu Arg Lys Leu Leu
 180 185 190
 Thr Gly Glu Leu Leu Thr Leu Ala Ser Arg Gln Gln Leu Ile Asp Trp
 195 200 205

Met Glu Ala Asp Lys Val Ala Gly Pro Leu Leu Arg Ser Ala Leu Pro
 210 215 220
 Ala Gly Trp Phe Ile Ala Asp Lys Ser Gly Ala Gly Glu Arg Gly Ser
 225 230 235 240
 Arg Gly Ile Ile Ala Ala Leu Gly Pro Asp Gly Lys Pro Ser Arg Ile
 245 250 255
 Val Val Ile Tyr Thr Thr Gly Ser Gln Ala Thr Met Asp Glu Arg Asn
 260 265 270
 Arg Gln Ile Ala Glu Ile Gly Ala Ser Leu Ile Lys His Trp
 275 280 285

(2) INFORMATION FOR SEQ ID NO: 11:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 207 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

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 1 5 10 15
 Leu Asn Glu Val Gly Ile Glu Gly Leu Thr Thr Arg Lys Leu Ala Gln
 20 25 30
 Lys Leu Gly Val Glu Gln Pro Thr Leu Tyr Trp His Val Lys Asn Lys
 35 40 45
 Arg Ala Leu Leu Asp Ala Leu Ala Ile Glu Met Leu Asp Arg His His
 50 55 60
 Thr His Phe Cys Pro Leu Glu Gly Glu Ser Trp Gln Asp Phe Leu Arg
 65 70 75 80
 Asn Asn Ala Lys Ser Phe Arg Cys Ala Leu Leu Ser His Arg Asp Gly
 85 90 95
 Ala Lys Val His Leu Gly Thr Arg Pro Thr Glu Lys Gln Tyr Glu Thr
 100 105 110
 Leu Glu Asn Gln Leu Ala Phe Leu Cys Gln Gln Gly Phe Ser Leu Glu
 115 120 125
 Asn Ala Leu Tyr Ala Leu Ser Ala Val Gly His Phe Thr Leu Gly Cys
 130 135 140
 Val Leu Glu Asp Gln Glu His Gln Val Ala Lys Glu Glu Arg Glu Thr
 145 150 155 160
 Pro Thr Thr Asp Ser Met Pro Pro Leu Leu Arg Gln Ala Ile Glu Leu
 165 170 175
 Phe Asp His Gln Gly Ala Glu Pro Ala Phe Leu Phe Gly Leu Glu Leu
 180 185 190
 Ile Ile Cys Gly Leu Glu Lys Gln Leu Lys Cys Glu Ser Gly Ser
 195 200 205

CLAIMS

1. A method for the determination of a tetracycline in a sample characterized in that
 - the sample is brought into contact with prokaryotic cells encompassing a DNA
 - 5 vector including a nucleotide sequence encoding a light producing enzyme under transcriptional control of a tetracycline repressor and a tetracycline promoter,
 - detecting the luminescence emitted from the cells, and
 - comparing the emitted luminescence to the luminescence emitted from cells in a control containing no tetracycline
 - 10 - wherein a detectable luminescence higher than a luminescence of the control indicates the presence of tetracycline in the sample.
2. The method according to claim 1 characterized in that the cells are *Escherichia coli*.
- 15 3. The method according to claim 1 or 2 characterized in that the DNA vector is a plasmid containing the luxCDABE genes (SEQ ID NO: 3), tetracycline repressor (TetR) (SEQ ID NO: 11) and tetracycline promotor (TetA) (SEQ ID NO: 9) from *Tn10*.
- 20 4. The method according to claim 3 characterized in that the DNA vector is the plasmid pTetLux1 (SEQ ID NO: 3).
5. The method according to claim 1 or 2 characterized in that
 - 25 - the DNA vector is a plasmid containing the insect luciferase gene (SEQ ID NO: 1), tetracycline repressor (TetR) (SEQ ID NO: 11) and tetracycline promotor (TetA) (SEQ ID NO: 9) from *Tn10*, and that

- D-luciferin is added to the mixture of the sample and the cells in order to initiate the luminescence of the cells.
6. The method according to claim 5 characterized in that the DNA vector is the
5 plasmid pTetLuc1 (SEQ ID NO: 1).
7. The method according to any of the claims 1 - 6 characterized in that the sensitivity of the analysis with respect to the tetracycline is controlled by
- increasing or decreasing the concentration of divalent metal ions, e.g.
10 magnesium ions, or
 - adjusting the pH, or
 - combined adjusting of the divalent metal ion concentration and the pH.
8. The method according to any of the claims 1 - 6 characterized in that the
15 sensitivity of the analysis with respect to the tetracycline derivative is increased by the use of cells which are especially antibiotic sensitive mutant strains.
9. The method according to any of the claims 1 - 8 characterized in that the luminescence is measured using an X-ray or polaroid film, a CCD-camera, a liquid
20 scintillation counter or a luminometer.
10. The method according to any of the claims 1 - 9 characterized in that the sample to be analyzed is milk, fish, meat, infant formula, eggs, honey, vegetables, serum, plasma, whole blood or the like.
- 25
11. A recombinant prokaryotic cell characterized in that it encompasses a DNA vector including a nucleotide sequence encoding a light producing enzyme, tetracycline repressor and tetracycline promoter.

12. The cell according to claim 11 characterized in that the DNA vector is a plasmid containing either

- the luxCDABE genes (SEQ ID NO: 3), tetracycline repressor (TetR) (SEQ ID NO: 11) and tetracycline promotor (TetA) (SEQ ID NO: 9) from *Tn10*, or
- the insect luciferase gene (SEQ ID NO: 1), tetracycline repressor (TetR) (SEQ ID NO: 11) and tetracycline promotor (TetA) (SEQ ID NO: 9) from *Tn10*.

13. The cell according to claim 11 or 12 characterized in that it is *Escherichia coli*.

10

14. The cell according to claim 12, 13 or 14, characterized in that it is in dried form, e.g. in lyophilized form.

15. A plasmid characterized in that it comprises either

- the luxCDABE genes (SEQ ID NO: 3), tetracycline repressor (TetR) (SEQ ID NO: 11) and tetracycline promotor (TetA) (SEQ ID NO: 9) from *Tn10*, or
- the insect luciferase gene (SEQ ID NO: 1), tetracycline repressor (TetR) (SEQ ID NO: 11) and tetracycline promotor (TetA) (SEQ ID NO: 9) from *Tn10*.

20 16. A plasmid according to claim 15 characterized in that it is pTetLux1 (SEQ ID NO: 3) or pTetLuc1 (SEQ ID NO: 1).

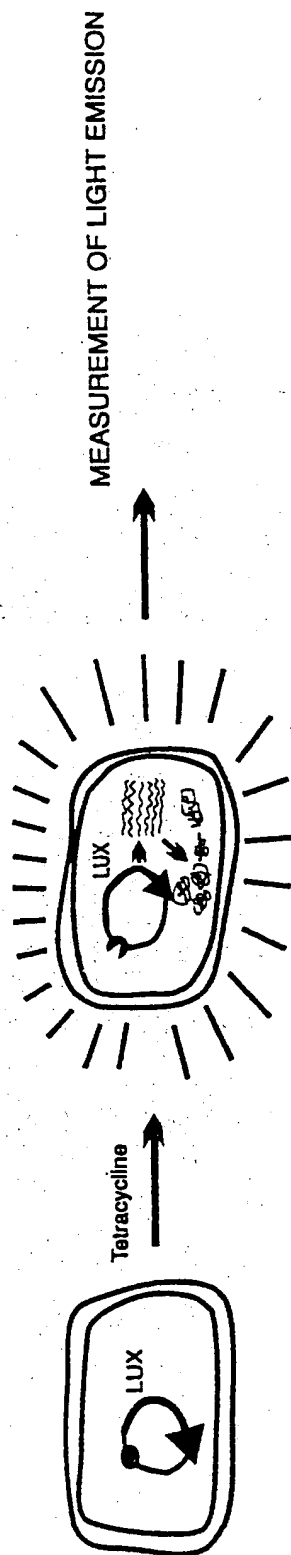


FIG. 1a

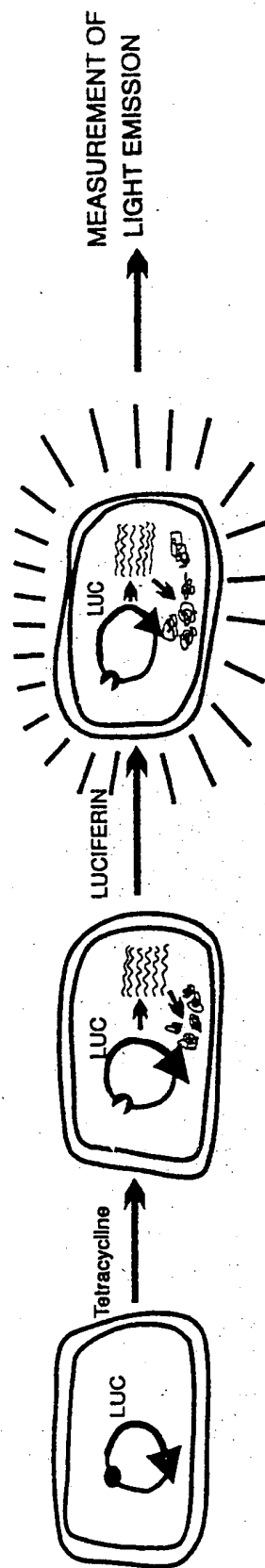


FIG. 1b

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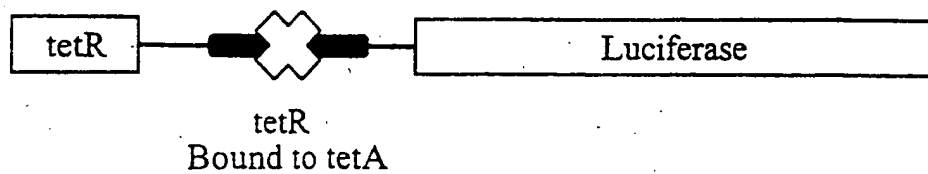
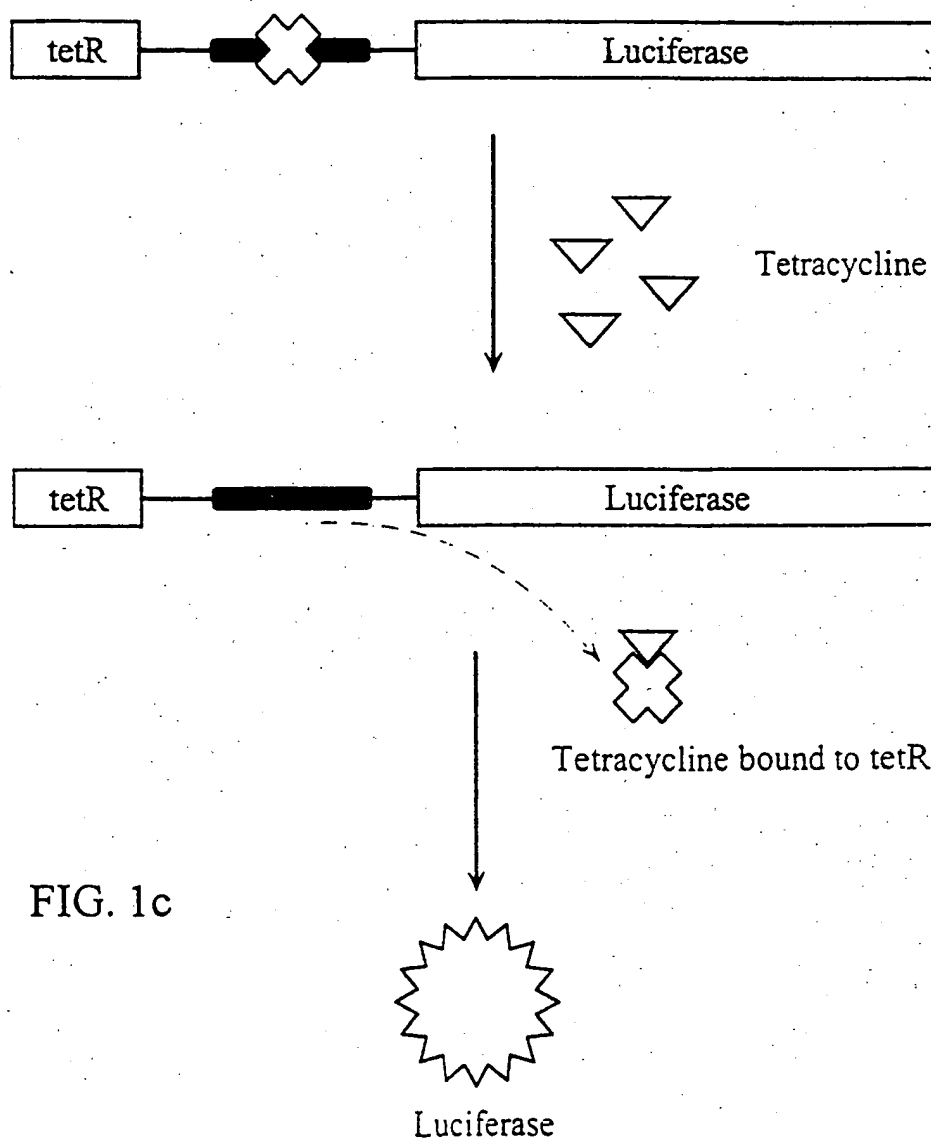
A. No Protein ExpressionB. Protein Expression

FIG. 1c

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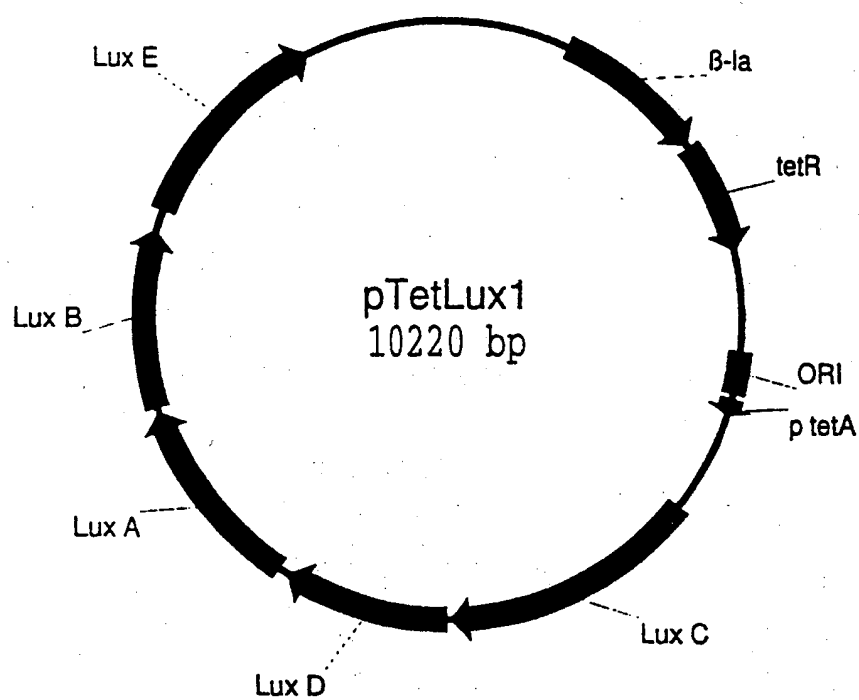


FIG. 2

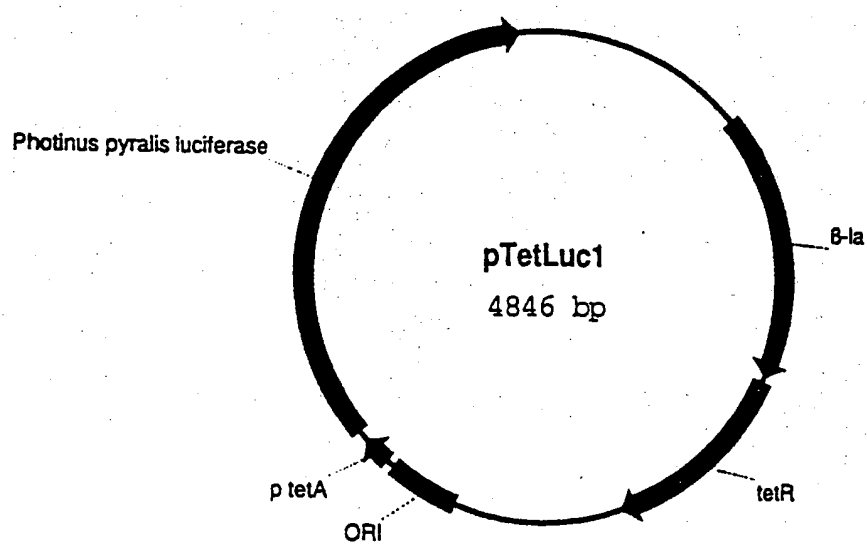


FIG. 3

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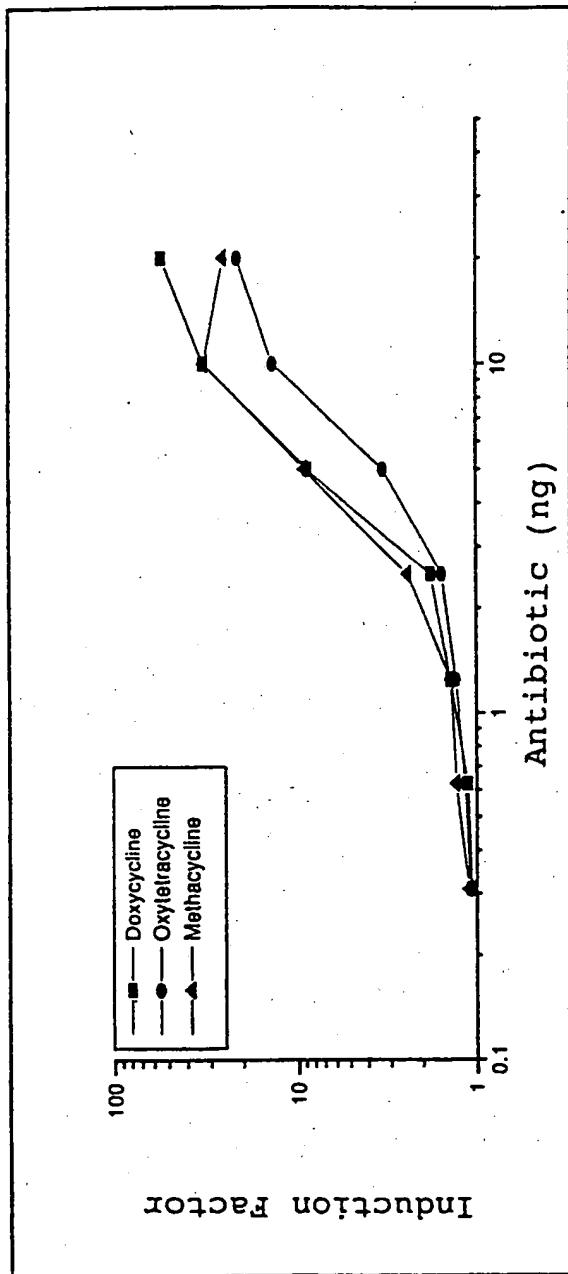


FIG. 4a

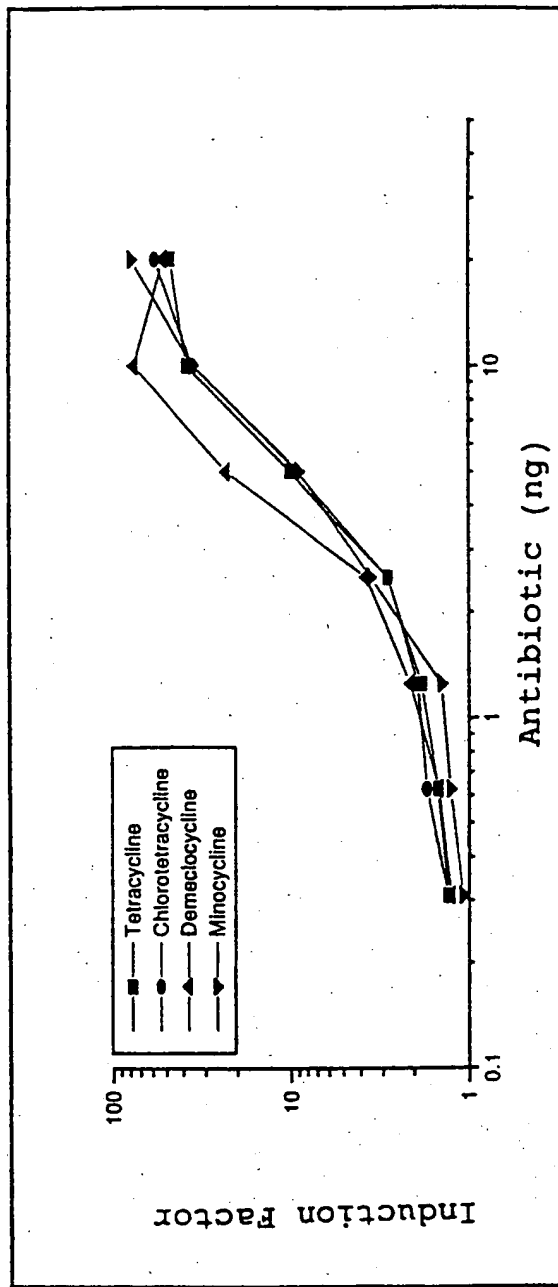


FIG. 4b

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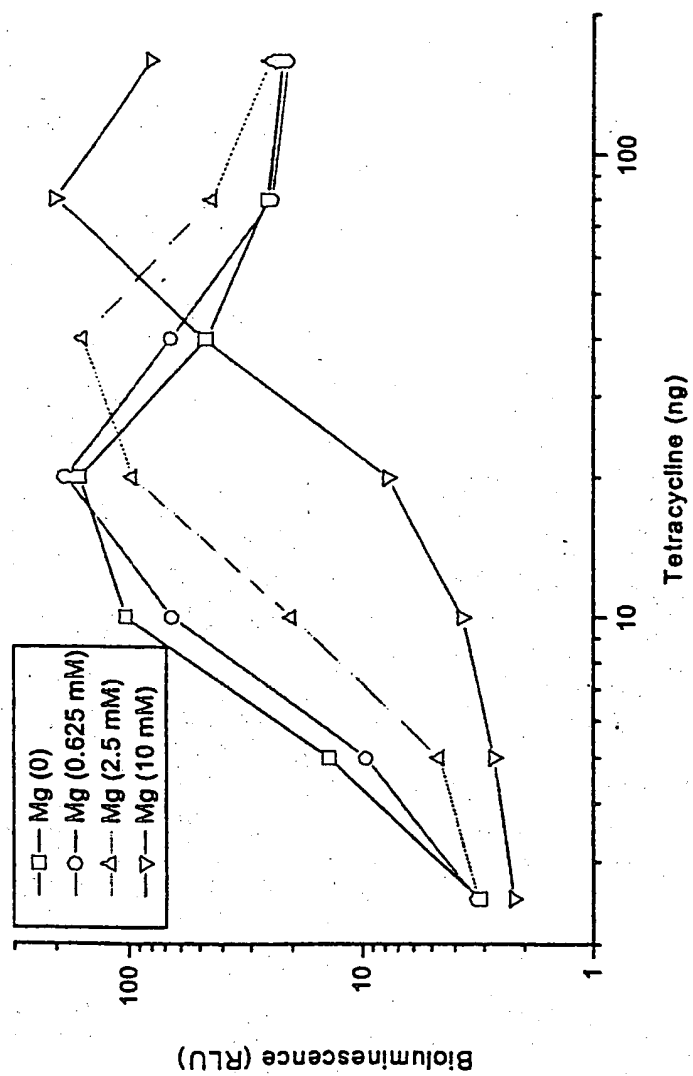


FIG. 5

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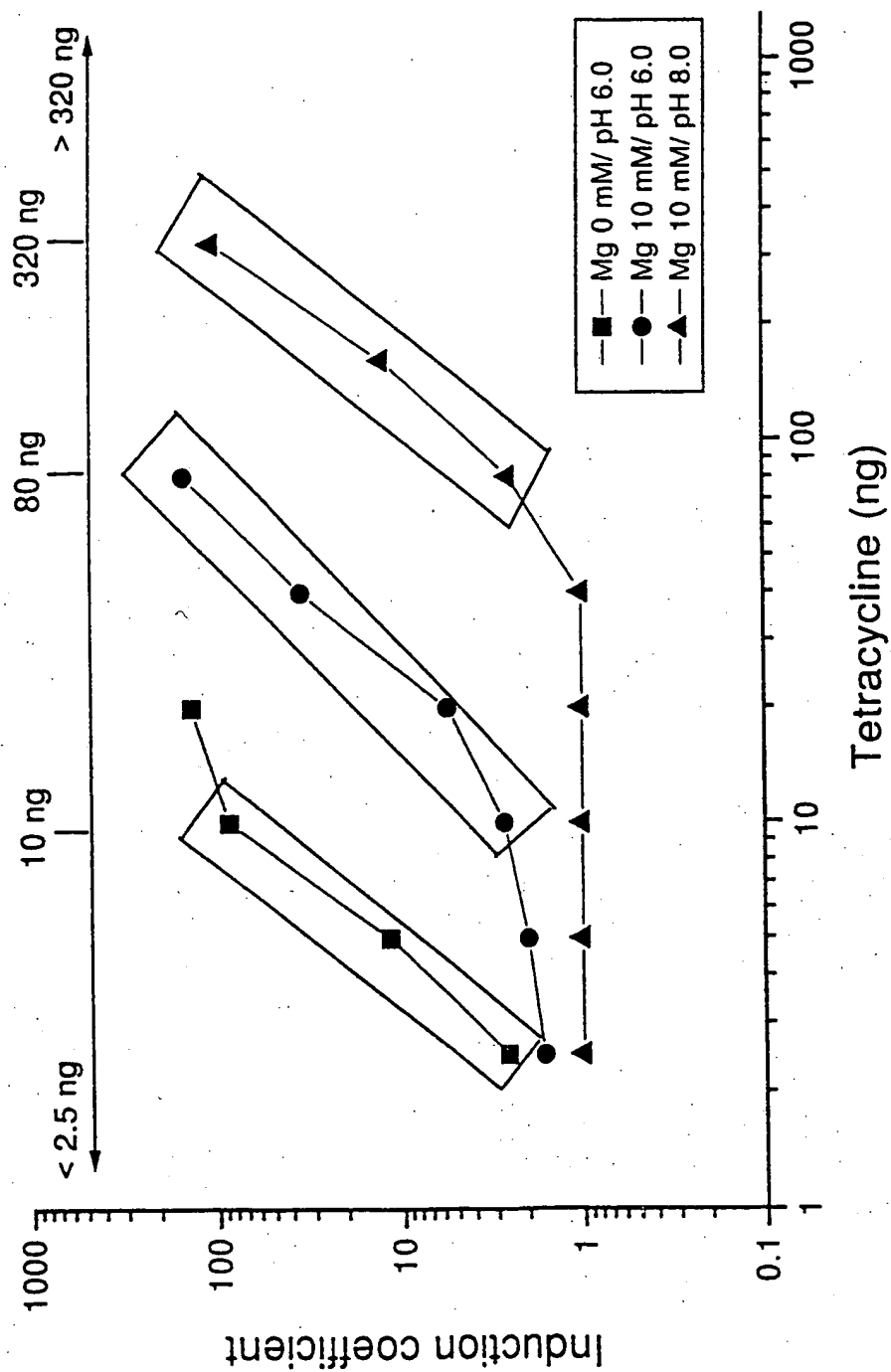


FIG. 6

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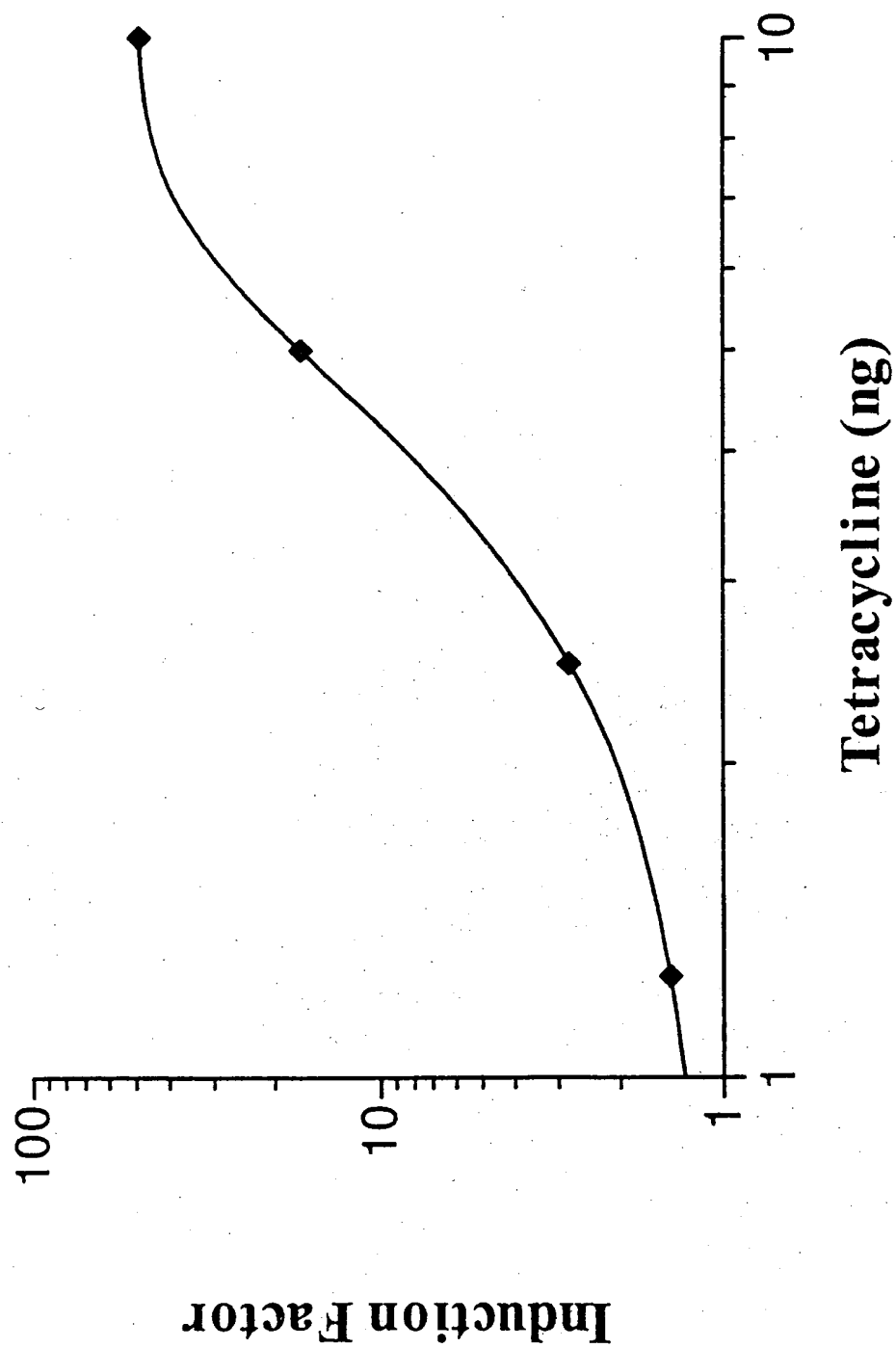


FIG. 7

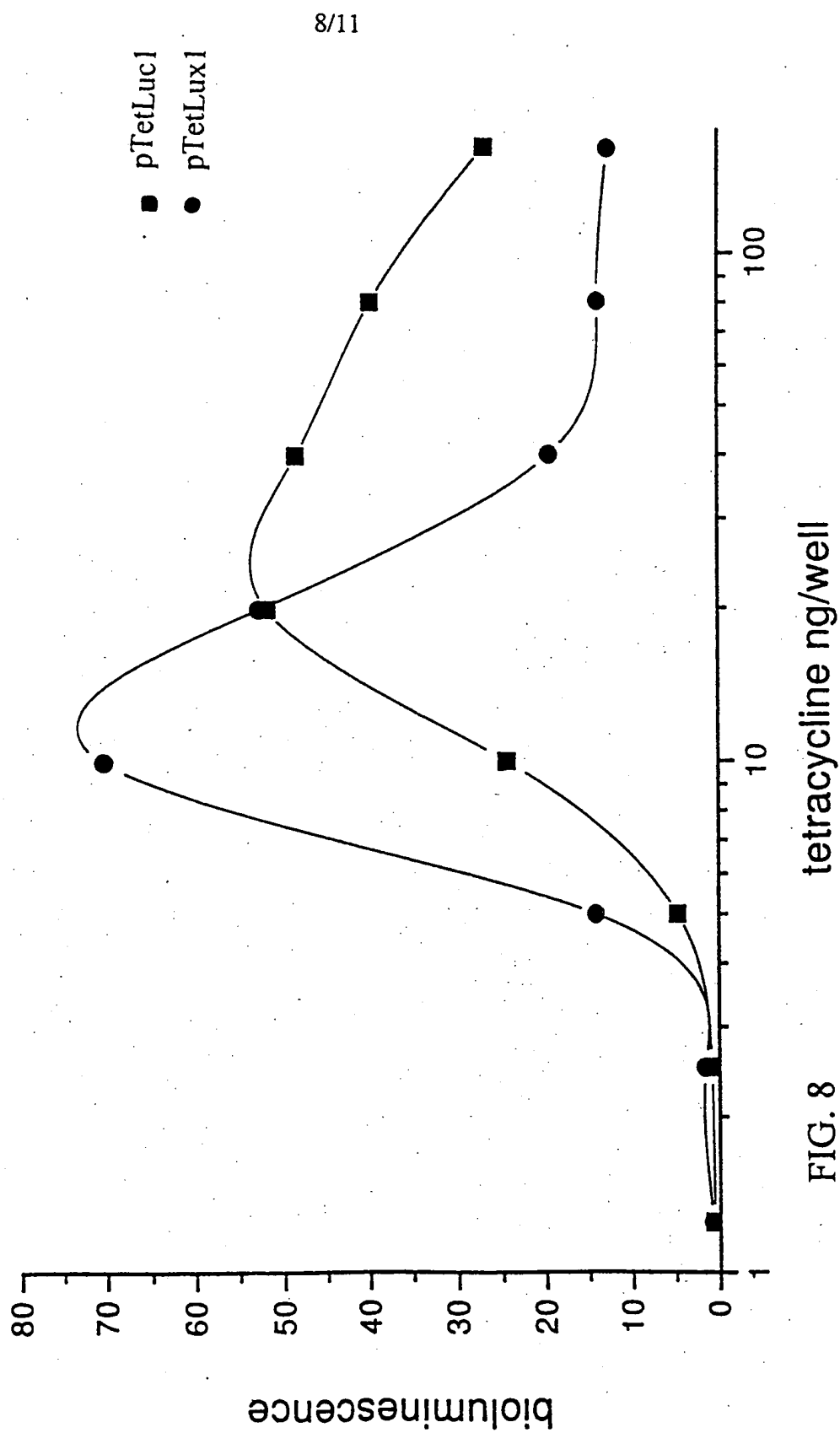


FIG. 8 tetracycline ng/well

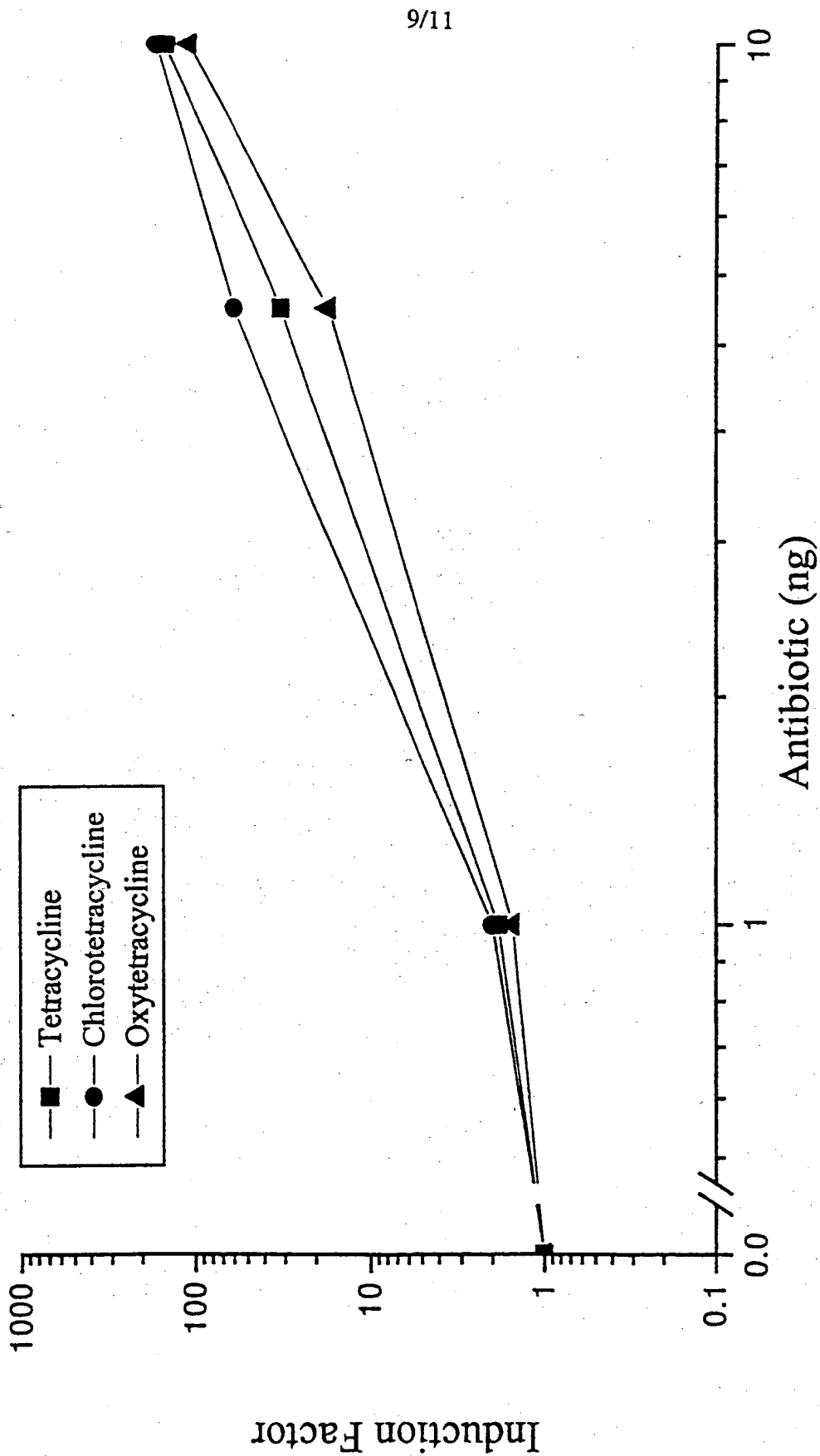


FIG. 9

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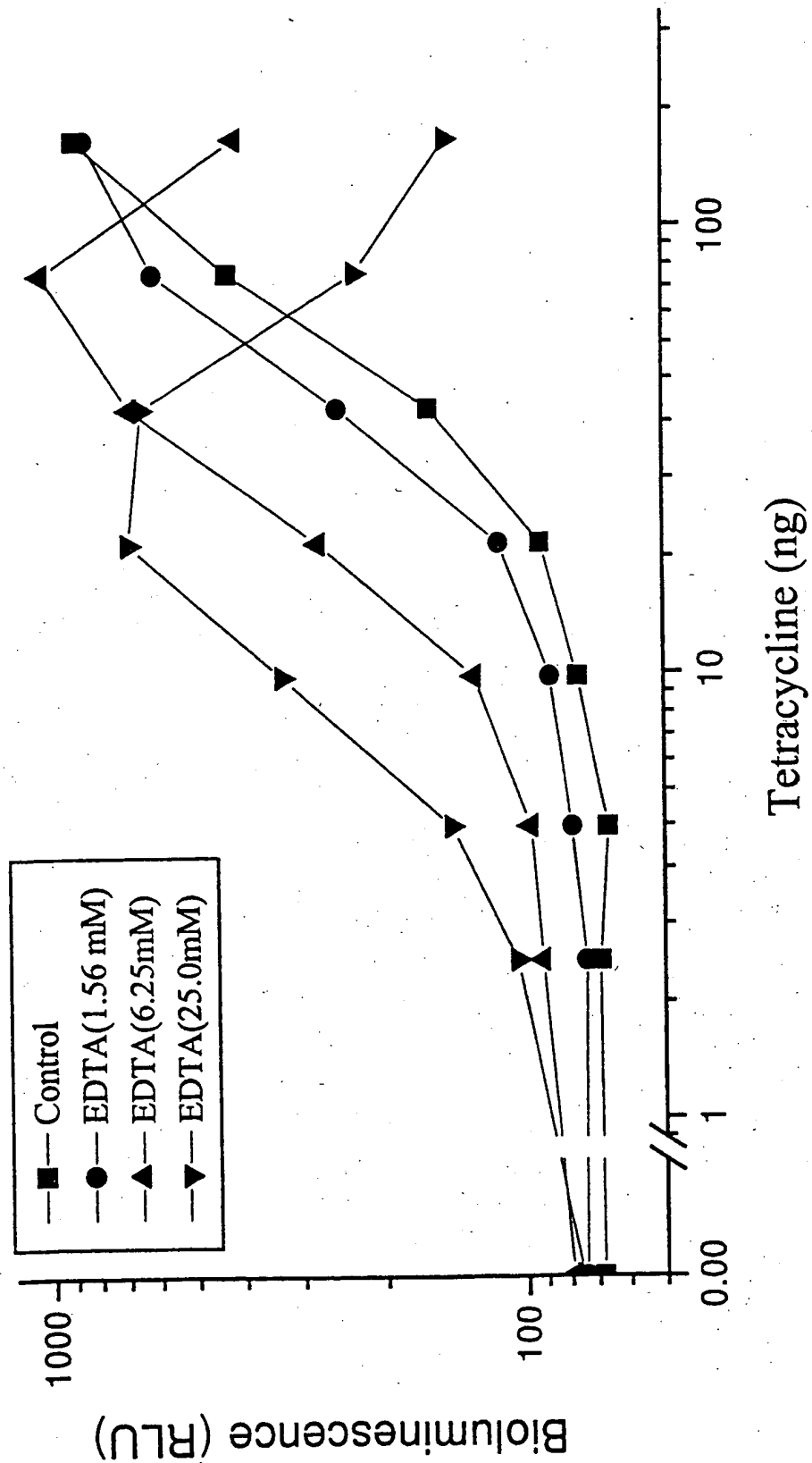


FIG.10

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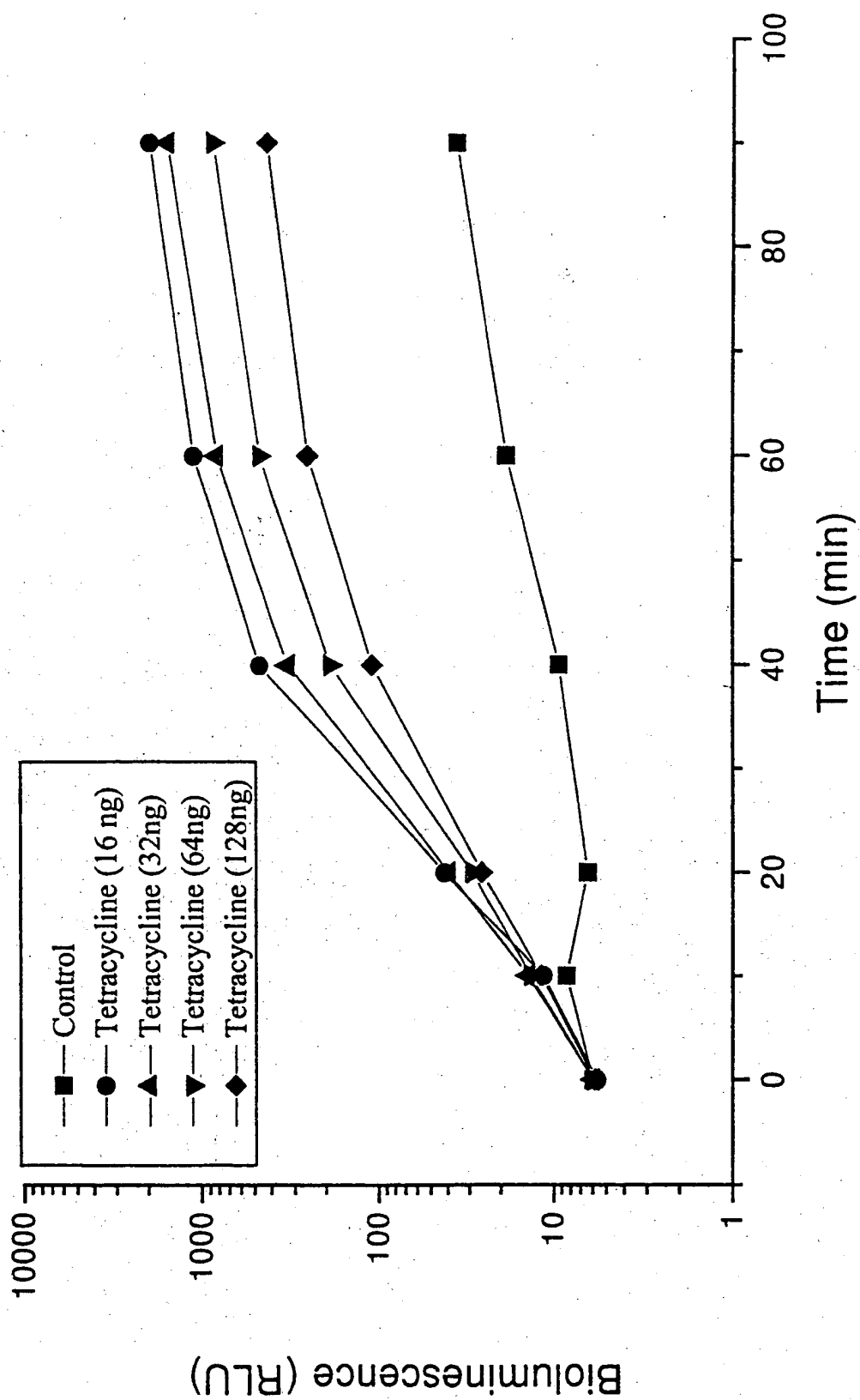


FIG.11

INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI 98/00873

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12Q 1/66, C12Q 1/18, C12N 1/21, C12N 15/53
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12Q, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, EPODOC, PAJ, MEDLINE, BIOSIS, CA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Nucleic Acids Research, Volume 25, No 6, March 1997, Rolf Lutz et al, "Independent and tight regulation of transcriptional units in Escherichia coli via the LacR/O, the TetR/O and AraC/I1-I2 regulatory elements", page 1203 - page 1210, See the entire article --	1-16
A	WO 9303179 A1 (BIO-TECHNICAL RESOURCES), 18 February 1993 (18.02.93), See esp. page 10, line 14-25 --	1-16

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

17 February 1999

Date of mailing of the international search report

27 -02- 1999

Name and mailing address of the ISA/
Swedish Patent Office
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Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.

PCT/FI 98/00873

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>Dialog Information Service, File 155, Medline, Dialog accession no. 08106145, Medline accession no. 95129845, Skerra A: "Use of the tetracycline promoter for the tightly regulated production of a murine antibody fragment in Escherichia coli", Gene (NETHERLANDS) Dec 30 1994, 151 (1-2) p131-5</p> <p>-- -----</p>	1-16

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/FI 98/00873

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9303179 A1	18/02/93	AU 2422792 A	02/03/93
		CA 2114103 A	18/02/93
		EP 0597984 A	25/05/94
		JP 6509712 T	02/11/94
		US 5571722 A	05/11/96
		US 5612184 A	18/03/97
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